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Effect of 2-Dimethylaminoethanol on Renal Hemodynamics.* (23920)

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Recently Pfeiffer *et al.*(1) reported that 2-dimethylaminoethanol (DMAE) may act as a possible tertiary amine precursor of acetylcholine. This observation associated with its possible therapeutic effects on the central nervous system led to this study. If DMAE acts as a precursor it might be expected to produce changes in urine flow and renal hemodynamics similar to those noted with acetylcholine. This report is concerned with the effect of DMAE[†] on urine flow in dogs and rats as well as hemodynamic changes in dogs.

Methods. Male and female rats weighing between 240 and 400 g receiving no food and water for 18 hours were used. Prior to experiment 25 ml of tap water/kg body weight was administered *via* stomach tube to each animal. The compounds to be tested were dissolved in water immediately before hydration. The rats were placed in metabolism cages, 2/cage, and

urine collected 5 hours. Urine measurements were made on control rats administered only water as well as rats given DMAE and choline chloride in varying doses. In addition, the effect of DMAE on renal hemodynamics were carried out on trained unanesthetized female dogs in the postabsorptive state. One hour before each experiment the dogs were hydrated with 25 ml of water/kg of body weight. Inulin and para-aminohippurate (PAH) clearances were used as a measure of glomerular filtration rate (GFR) and renal plasma flow (RPF) respectively. Inulin was analyzed according to the method of Roe, Epstein and Goldstein(2) and PAH according to the method of Smith *et al.*(3). Throughout the experiment glucose 5% in water was infused at constant rate of 3 ml/min. Following 2 control periods, DMAE was infused for 1 hour followed by one hour during which time 2 more clearance samples were taken. Doses of DMAE infused were 1.0, 1.5, 2.5, 5.0 and 7.5 mg/kg.

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† Supplied by Dr. C. C. Pfeiffer, Emory University.

TABLE I. Effect of DMAE on Water Excretion in the Rat.

Rat No.	Water adm. (ml)	% of load excreted	Rat No.	Water adm. (ml)	% of load excreted
A. 25 ml water/kg			B. Choline, 75 mg/kg		
1, 22	14.35	72	15, 19	14.40	55
43, 4	16.00	68	42, 32	7.90	62
7, 11	9.50	43	15, 20	14.30	28
8, 19	7.83	51	1, 2	14.85	64
21, 22	16.30	62	8, 18	14.35	29
45, 26	15.85	47	10, 4	16.15	38
33, 41	17.00	38	15, 19	14.40	49
11, 13	14.30	63	42, 60	8.68	53
2, 19	13.80	58	4, 5	17.49	46
19, 43	15.40	71	4, 11	17.05	27
51, 32	12.60	42	2, 18	15.00	38
20, 30	9.50	58	8, 18	14.35	29
Avg % excreted of total load		56.08 $\pm$ 11.7			43.17 $\pm$ 13.5
C. DMAE, 50 mg/kg			D. DMAE, 75 mg/kg		
1, 3	16.00	22.5	8, 9	14.90	42
11, 13	15.70	53.4	2, 3	14.90	42
19, 18	14.85	43.7	1, 15	14.60	14
7, 8	15.55	38.5	40, 39	13.05	20
9, 10	12.00	48.0	34, 33	12.50	16
14, 15	13.50	8.1	35, 36	14.25	22.3
17, 20	12.30	23.6	1, 10	14.95	6.7
6, 17	10.80	20.3	14, 19	13.30	5.1
16, 20	11.25	40.0	8, 9	11.95	40
			8, 3	14.95	37
			48, 49	8.20	46
			53, 37	8.12	45
			9, 8	14.60	32
			4, 5	15.93	29
			18, 1	15.40	28
			19, 8	16.20	18
Avg % excreted of total load		33.12 $\pm$ 15.06			26.57 $\pm$ 13.0
		Diff. between means	t for diff.	P	
Water—Choline		12.91	2.49	<.05	
" —DMAE 50		22.96	3.93	<.001	
" — " 75		29.51	6.20	<.001	
Choline—DMAE 50		10.05	1.61	>.1	
" — " 75		16.60	3.29	<.01	
DMAE 50—DMAE 75		6.55	1.14	>.2	

Results. Table I indicates the effect of DMAE and choline chloride on urine flow in rats. Results are expressed as percent excreted of total amount of water administered. Compared to water, choline chloride produced a significant decrease in urine flow, ($P < 0.05$). DMAE at both dosage levels caused a greater decrease in urine flow ($P < 0.001$). In addition, it can be noted that choline chloride and DMAE (50 mg/kg) produced similar changes whereas DMAE (75 mg/kg) caused a significant decrease compared to choline chloride. These results indicate that in rats DMAE in doses employed, produced a significant anti-diuresis. Whether DMAE *per se* inhibited

water excretion through liberation of the anti-diuretic hormone or DMAE acted as a precursor of acetylcholine which then either released antidiuretic hormone or caused hemodynamic changes can not be ascertained from this study.

Since a decrease was noted in rats, it was anticipated that a similar action would occur in dogs if this were a specific effect of DMAE. Table II illustrates experiments employing 5.0 and 7.5 mg/kg doses to indicate lack of effect of DMAE on renal hemodynamics. Ten mg/kg of DMAE produced central nervous system effects of tremors, muscular twitching, emesis and defecation which limited studies

TABLE II. Effect of DMAE on Renal Hemodynamics.

Dog No.	Urine flow, ml/min.			G. F. R., ml/min.			R. P. F., ml/min.			Dose, mg/kg
	C	I	R	C	I	R	C	I	R	
1	2.41	2.88	3.08	58.5	62.2	56.0	195	209	185	5
2	4.00	3.47	2.72	71.0	59.0	67.1	238	198	190	5
3	2.71	2.76	3.04	48.8	48.3	47.2	138	121	113	5
3	3.64	2.33	2.45	58.6	44.3	49.5	125	115	134	5
2	3.03	3.15	2.95	68.0	66.2	69.1	215	225	214	7.5
2	4.31	3.95	4.12	64.0	67.3	65.2	231	240	229	"
3	2.95	3.08	3.30	54.2	53.4	58.6	140	131	135	"
4	4.00	3.90	3.98	59.0	54.0	61.2	195	208	212	"
5	3.60	3.80	3.51	53.0	56.0	54.2	185	190	178	"

at this concentration. On the basis of these results in dogs it would appear that DMAE in doses that are well tolerated does not have any specific effect on the kidney in acute experiments. No changes in sodium, potassium or chloride excretion were noted in these experiments.

Summary. Renal effects of DMAE were determined in rats and dogs. In high doses an antidiuretic effect was noted in rats. Dogs that had been administered DMAE in maximal tolerated doses exhibited no changes in urine flow nor renal clearances. There were

no indications that this compound had any toxic effect on the kidney.

Statistical analysis kindly performed by E. H. Jenny, Emory University.

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Metabolism of Cortisol by Loose Connective Tissue *in vitro*. (23921)

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Extensive transformation of progesterone (1) and cortisol(2) (4-pregnene-11-beta, 17-alpha, 21-triol, 3, 20-dione, or hydrocortisone) to a series of metabolites by eviscerated rats has been demonstrated. These metabolites represent a pool of steroids which have been transformed by a variety of tissues. The extent to which each tissue adds to the pool of steroid products is being investigated. Connective tissue obtained from mice was incubated *in vitro* in the presence of cortisol-4-C¹⁴. Histological examination of this tissue has shown that 90% of the cells are fibroblasts(3).

Various products were isolated from these incubations and the following 5 compounds were identified: 4-pregnene-11-beta, 17-alpha,

20-alpha, 21-tetrol, 3-one (20-epi substance "E" of Reichstein); 4-pregnene 17-alpha, 21-diol, 3, 11, 20-trione (cortisone); pregnane 11-beta, 17-alpha, 21-triol, 3, 20-dione (dihydrocortisol); 4-pregnene 11-beta, 21-diol, 3, 20-dione (corticosterone) and 4-androstene 11-beta-ol, 3, 17-dione.

Materials and methods. Fourteen mice (AK) were killed by decapitation and the connective tissue collected as described previously(4). The areolar tissue was placed in 8 incubation flasks immediately after collection. Each flask contained 0.3-0.6 g of tissue in 10 ml of 0.1 M phosphate buffer, pH 7.4 and cortisol 4-C¹⁴ (25,000 cpm; Specific Activity 1.467 mc/mole). Incubation was carried out in shaking water bath at 37°C for a total of

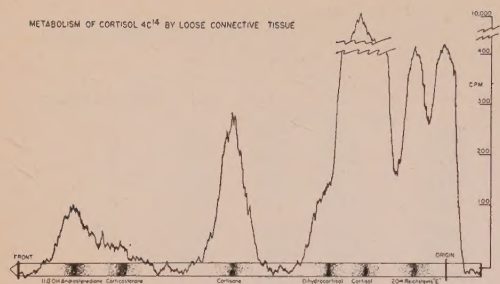


FIG. 1. Radiochromatographic tracing, chloroform/formamide system.

4 hours. Two control flasks were incubated without tissue and 2 other zero hrs. controls with tissue were not incubated. The contents of each flask were extracted 3 times with 60 ml of hot acetone. The acetone was subsequently filtered and evaporated under a stream of nitrogen. The aqueous phase remaining after evaporation of acetone, was then extracted 4 times with 30 ml portions of chloroform. The chloroform fraction was evaporated and directly chromatographed in the chloroform/formamide system of Zaffaroni(5). Radioactive analysis of the chromatograms, identification and determination of specific activities of the different metabolites was carried out as described in detail previously(2,6,7).

Results. Seven zones of radioactivity were detected from the original chromatograms obtained from chloroform/formamide system in each of the incubated connective tissue experiments. The Rf's of the radioactive peaks and shoulders were: .00, .08, .19, .27, .51, .76 and .88 (Fig. 1). Rechromatography of the origin and high polar region in chloroform formamide for 12 hrs. was composed of 2 peaks. After acetylation(6) and rechromatography in the benzene/formamide system for 3 hr the zones of Rf's .19 and .29 were resolved into 3 radioactive compounds. Two radioactive peaks could be separated from the low polar region (Rf's .76 and .88) after rechromatography in the benzene/formamide system for 5 hr. From incubated and non-incubated control flasks only cortisol was isolated. Identification of the isolated steroids was based on the following data:

Cortisol. To an aliquot obtained from the peak of Rf .19 of the original chromatogram,

100 μ g of non-radioactive carrier cortisol were added and the mixture acetylated and chromatographed in benzene/formamide for 6 hrs. The carrier and radioactive peak migrated at the same rate. At this time the specific activity was determined (32.1 cpm/ μ g). The mixture was oxidized(6) and rechromatographed in benzene/formamide for 4 hr. The oxidized mixture (cortisone acetate) had a specific activity of 32.3 cpm/ μ g.

4-pregnene 11-beta, 17-alpha, 20-alpha, 21-tetrol, 3-one (20-epi Substance "E" of Reichstein). Standard 20-epi substance "E" and the radioactive peak (Rf 0.08, Fig. 1), occurred at the same rate in chloroform/formamide for 8 hr (S.A. 12.8 cpm/ μ g). Acetylation of the mixture and chromatography in the benzene/formamide system for 9 hr demonstrated that both acetylated compounds migrated at the same rate (S.A. 12.7 cpm/ μ g). Oxidation of the acetylated derivatives gave as final product 4-pregnene 17-alpha, 20-alpha, 21-triol, 3, 11-dione, 20, 21-diacetate (S.A. 12.9 cpm/ μ g). When carrier Substance "E" of Reichstein (20-beta-ol form), was chromatographed with an aliquot obtained from the original high polar region, the specific activity of the mixture dropped from 14.1 cpm/ μ g to 0.1 cpm/ μ g after acetylation and no radioactivity could be detected in this carrier after oxidation.

Pregnane 11-beta, 17-alpha, 21-triol, 3, 20-dione (Dihydro-cortisol). The radioactive zone obtained from the region of Rf 0.27 (Fig. 1), was mixed with non-radioactive carrier dihydro-cortisol, the mixture was rechromatographed in the "free," acetylated and oxidized states. In every case carrier and radioactivity migrated at the same rate. Specific activities were not determined on this compound.

4-pregnene 17-alpha, 21-diol, 3, 11, 20-trione (Cortisone). The radioactivity in the cortisone region (Rf 0.51) was eluted and divided into 2 equal portions. To each portion was added 100 μ g of nonradioactive cortisone (S.A. 36.6 cpm/ μ g). One portion was oxidized and then chromatographed in benzene/formamide. The Rf (0.5) and the specific activity (36.3 cpm/ μ g) indicated that the mixture was oxidized to 4-androstene 3, 11,

17-trione (Adrenosterone). The other portion was acetylated and chromatographed in benzene/formamide for 8 hr, the specific activity of the derivative (cortisone acetate) was 36.6 cpm/ μ g.

4-pregnene 11-beta, 21-diol, 3, 20-dione (Corticosterone). The radioactive unknown (Rf 0.76, Fig. 1) was combined with 300 μ g of non-radioactive corticosterone and chromatographed in the chloroform formamide system for 3 hr. The specific activity of the mixture was 6.3 cpm/ μ g. The eluted mixture was divided into 3 aliquots. The first aliquot was acetylated and chromatographed in benzene/formamide for 3 hours; the derivative corticosterone acetate had a specific activity of 6.6 cpm/ μ g. This same aliquot was subsequently oxidized and chromatographed in the hexane-benzene/formamide system for 4 hr. The derivative 11-dehydro-corticosterone acetate had a specific activity of 6.5 cpm/ μ g. The second aliquot was mildly oxidized with 1 mg of CrO_3 in 15 drops of glacial acetic acid for 3 minutes at 80°C and chromatographed in benzene/formamide for 10 hr. This type of oxidation will give a 45% yield of 11-dehydro-corticosterone. The specific activity of this derivative was 6.6 cpm/ μ g. The third aliquot was strongly oxidized with 4 mg of CrO_3 in 15 drops of glacial acetic acid for 3 hr at 80°C and chromatographed in chloroform/formamide for 35 hr. This type of oxidation will give a 30-40% yield of the etioacid of 11-dehydro-corticosterone. The specific activity of this derivative was 6.8 cpm/ μ g.

4-androstene 11-beta-ol, 3, 17-dione. To the eluate obtained from the zone of lower polarity (Rf 0.88), 150 μ g of the carrier non-radioactive compound was added and rechromatographed in benzene/formamide for 3 hr. The specific activity of the mixture was 32.3 cpm/ μ g. Oxidation of the eluted mixture and rechromatography in the same system gave as final product adrenosterone, with a specific activity of 32.6 cpm/ μ g.

Unknown compounds. At the present time a highly polar compound isolated from the original chromatogram is under study. One of the compounds obtained after acetylation of the region with Rf's 0.19 and 0.27, behaved chromatographically as a diacetate, which re-

sembled 4-pregnene 17-alpha, 20-alpha, 21-triol, 3, 11-dione; however, the carrier was not available at this time in our laboratory for the actual identification of this steroid.

Discussion. Numerous studies have been reported concerning metabolism of cortisol and other steroids by the liver(8). The major metabolic changes of cortisol appear to be loss of the side chain, oxidation reduction at 3, 4, 11 and 20 positions and formation of water soluble conjugates(8,9). Kidney homogenates(10) have also been reported to metabolize cortisol in a similar fashion. Leukemic cells(11) have the capacity to alter the chemical structure of this hormone in a cyclic manner. It has been reported that osteosarcomas can remove the 17-alpha hydroxyl group of cortisol to form corticosterone(12). That the liver is not the main or only organ capable of transforming cortisol and other steroid hormones to various metabolites has been further demonstrated by the experimental use of eviscerated rats(1,2).

In our experiments it is demonstrated that cortisol can be changed chemically by connective tissue. The molecular changes include oxidation of the hydroxyl group in position C-11, reduction of the 4-5 double bond, reduction of the C-20 ketone (C-20-alpha-ol form) and oxidative cleavage of the side chain. A particularly interesting reaction is the removal of the 17-alpha hydroxyl group to form corticosterone. It has been proven in our laboratory that human tissue culture fibroblasts metabolize cortisol in a similar fashion except that the isomerism of reducing the C-20 ketone by these cells is in the 20-beta form(13). Formation of corticosterone has also been confirmed in these experiments(13). The possibility that removal of the C-17 hydroxyl group could be an artefact(14) was eliminated by showing that the extracts of control flasks contained only cortisol.

The predominant cell found in connective tissue is the fibroblast(3). Histological, histochemical and radioautographic studies(4, 15) have shown that fibroblasts treated with cortisol round up and in this state resist destruction in areas of inflammation and it has been demonstrated that radioactive cortisol

tends to localize mainly either at the surface of fibroblasts or within them. It has been suggested that this hormone-induced change in fibroblasts enhances their resistance to cellular damage and thus interrupts a chain reaction of cellular destruction which potentiates inflammation(16,17). The relation of this increased resistance and the ability of this cell to transform the chemical structure of cortisol may be related to its mechanism of anti-inflammatory action. This is further suggested by the fact that none of the conversion products resulting from fibroblastic action on cortisol have anti-inflammatory activity(4). It therefore appears that it is cortisol itself which possesses anti-inflammatory activity. Since cortisol is at least 7 or 8 times more potent in its antiphlogistic effect than cortisone(3) and it has been demonstrated that cortisone and cortisol are inter-convertible(8), it is very possible that cortisol only is anti-inflammatory and that cortisone must be converted to cortisol for this hormone to be antiphlogistic. This possibility explains the difference in potency of these two compounds.

Summary. Action of the loose connective tissue (90% of cells are fibroblasts) on cortisol was investigated. Data are presented on the isolation and identification of 5 of the 7 metabolites observed when cortisol-4-C¹⁴ was incubated with mouse (AK) loose connective tissue. The identified metabolites are: 20-epi substance E of Reichstein; cortisone; dihydro-cortisol; corticosterone; and 4-androstene, 11-beta-ol, 3, 17-dione. The relationship of this metabolic picture to the fibroblastic role

on the anti-inflammatory action of cortisol is discussed.

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Role for Pancreas in Biosynthesis of Creatine.* (23922)

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Two enzymes are involved in the biosynthesis of creatine from its amino acid precursors: arginine-glycine transamidinase and guanidinoacetate methyltransferase(1,2). It has been generally accepted that arginine-glycine transamidination occurs only in the kidney; a product of this reaction, guanidinoacetate (glycocyamine), is then methylated in the liver to form creatine. Several mammalian tissues other than kidney have been examined in the past for transamidinase activity, but the results have been uniformly negative (1,3). Recently, in an attempt to clarify the mechanism of transamidinase action, this laboratory undertook a search for other sources of the enzyme. One result of this search was the discovery in *Streptomyces griseus* of a new transamidinase which, unlike kidney transamidinase, does not react with glycine (4). A second result of this search was the discovery of a transamidinase which occurs in high concentration in mammalian pancreas; in this paper certain characteristics of pancreatic transamidinase will be described.

Methods. Dog pancreas and kidneys were removed from the carcass as soon as possible and stored in the frozen state. Ten % tissue homogenates were prepared with a Ten Broeck glass homogenizer in 0.1 M phosphate buffer, pH 7.4, containing 5 mg/ml of ethylenediamine tetraacetic acid (Versene). A more purified enzyme preparation was obtained from pancreas by centrifuging the homogenate and fractionating the supernatant solution with ammonium sulfate at 4°C. The fraction precipitating between 30% and 60% saturation was taken up in buffer plus Versene, dialyzed for 18 hours at 4°C against dilute buffer-Versene, and stored in the frozen state. In the deproteinized incubation mixtures arginine was estimated by the Sakaguchi reaction(5) and hydroxyguanidine was as-

sayed by a pentacyanoammonioferrate method(4). Incubations were carried out in small test tubes in 0.1 M phosphate buffer, pH 7.4. Canavanine sulfate and hydroxylamine hydrochloride were neutralized before use with potassium hydroxide. Synthetic hydroxyguanidine sulfate, used as a standard in the colorimetric assays, was prepared from 1 M S-methylisothiuronium sulfate and 1.5 M hydroxylamine (prepared from hydroxylamine sulfate plus barium hydroxide). The aqueous solution was allowed to react for 24 hours at room temperature in a hood. Hydroxyguanidine sulfate was then precipitated from the reaction mixture by the slow addition of acetone; the filtered precipitate was washed with methanol, followed by acetone. The product was recrystallized from water. The structure of hydroxyguanidine was confirmed by elementary analysis and by reduction to guanidine with palladium and hydrogen. The pK of hydroxyguanidine was found to be approximately 8.1; it is stable in acid solution, but it is less stable in neutral and basic solutions.

Results. Preliminary experiments established that pancreatic transamidinase has the same substrate specificity as kidney transamidinase. Canavanine, arginine, and guanidinoacetate are the most active amidine donors, while ornithine, canaline, and glycine are the most active amidine acceptors(6). It would appear that the synthesis of guanidinoacetate is a major function of pancreatic transamidinase.

Attention was next directed to a comparison of enzyme activities in kidney and pancreas. For this purpose a choice of assay systems from among the various donor-acceptor pairs had to be made. It was recognized that for enzymic rate studies irreversible reactions would be preferable. Previous work in this laboratory had indicated that all transamidinases, whatever their source, have the ability to catalyze (a) an arginine-ornithine exchange reaction(7), and (b) a transfer of the

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TABLE I. A Comparison of Transamidinase Activities of Kidney and Pancreas Homogenates.

Assay system	μ moles product formed/g tissue (wet wt)/hr	
	Kidney	Pancreas
(a) Canavanine, .032 M Ornithine, .022 M Acetone, .4 M 37 mg tissue/ml; 1 hr at 35°C	27	132 (arginine)
(b) Arginine, .033 M Hydroxylamine, .6 M 50 mg tissue/ml; 2 hr at 25°C	2.1	13.3 (hydroxyguanidine)

amidine group of arginine to hydroxylamine, with the formation of hydroxyguanidine and ornithine(8). For assay purposes in this laboratory canavanine, a structural analogue of arginine, is substituted for arginine in Reaction (a). The resulting reversible canavanine-ornithine transamidination can then be made essentially irreversible by trapping one of the reaction products, canaline, with a carbonyl compound such as acetone or pyruvate(9); the other reaction product is arginine, which can be determined colorimetrically. Alternatively transamidinase activity can be assayed by Reaction (b), provided that the system assayed has relatively little arginase activity, since as will be seen, ornithine strongly inhibits Reaction (b).

In Table I the transamidinase activities of dog kidney and dog pancreas are compared, using both assay systems. It is evident that

on a wet weight basis transamidinase activity is approximately 5 times higher in pancreas than in kidney. Approximately the same ratio of activities was also obtained for canavanine-glycine transamidination, as estimated from guanidinoacetate formation.

In studies of the mechanism of transamidinase action carried out in this laboratory, attention has recently been focussed upon the role of hydroxylamine as an amidine acceptor. It has been suggested that hydroxylamine might react nonenzymatically with an enzyme-amidine intermediate formed by the interaction of arginine with transamidinase(4). In any event it can be seen from Fig. 1 that high concentrations of hydroxylamine are required to achieve a maximal rate of arginine-hydroxylamine transamidination. (For the experiments of Fig. 1-3, a dialyzed ammonium sulfate enzyme fraction from dog pancreas was used, instead of the more complex homogenate system.) Fig. 2 and Fig. 3 summarize the results obtained from time-course studies of arginine-hydroxylamine and guanidinoacetate-hydroxylamine transamidination reactions, respectively. Both reactions are strongly and specifically inhibited by the amidine acceptors ornithine and glycine. Other closely related compounds which are not amidine acceptors are also not effective inhibitors of hydroxyguanidine formation(4,8).

Discussion. The observation that pancreas is a rich source of arginine-glycine transami-

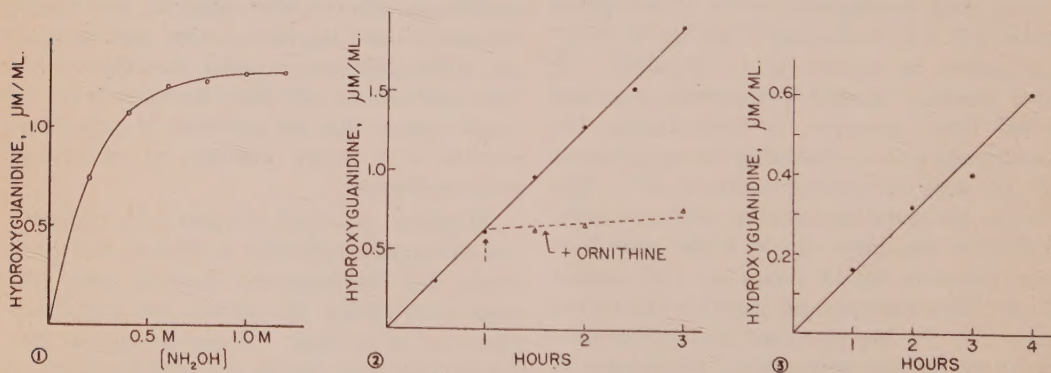


FIG. 1. Rate of arginine-hydroxylamine transamidination as a function of hydroxylamine concentration. Arginine, .033 M; 3 mg enzyme/ml; incubated 90 min. at 21°C.

FIG. 2. Time-course of arginine-hydroxylamine transamidination, showing inhibition by a competing amidine acceptor added to an aliquot at the indicated time. Arginine, .033 M; hydroxylamine, .6 M; ornithine, .03 M; 3 mg enzyme/ml; incubated at 21°C.

FIG. 3. Time-course of guanidinoacetate-hydroxylamine transamidination. Guanidinoacetate, .02 M; hydroxylamine, .6 M; 10 mg enzyme/ml; incubated at 25°C.

dinase suggests that this organ plays an important role in the biosynthesis of body creatine. Whether or not methylation of guanidinoacetate also occurs in pancreas remains to be determined. In any event guanidinoacetate formed in the pancreas and released into the blood stream would have but a short journey *via* the portal vein to the liver, where methylation definitely can take place(2). In contrast to pancreas, the weak transaminidase activities of thymus and testis, briefly noted elsewhere(4), may be too low to be of physiological significance. It is of interest to compare the distribution of transaminidase in the mammal with that of another enzyme involved in arginine metabolism, argininosuccinase, previously known to occur in mammals only in liver and kidney(10). Argininosuccinase has recently been found in this laboratory to occur in numerous other tissues of the body, such as brain, testis, spleen, pancreas, thymus, heart, and skeletal muscle. The significance of this distribution is not known at present.

Summary. Arginine-glycine transaminidase, one of the 2 enzymes concerned with the biosynthesis of creatine from amino acid precursors, has been found to occur in high concentration in mammalian pancreas. Homo-

genates of dog pancreas have approximately 5 times the transaminidase activity of kidney homogenates from the same animal, on a wet weight basis. The assay systems by which these tissues were compared included (a) canavanine-ornithine transamination, made unidirectional by the addition of a carbonyl compound to trap canaline, and (b) arginine-hydroxylamine transamination. It is suggested that the ability to catalyze these latter two reactions is a characteristic property of transaminidases. Chemical and enzymic syntheses of hydroxyguanidine are described.

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Effect of Cortisol on Gastric Ulcers of the Shay Rat. (23923)

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Since Shay *et al.*(1) published that gastric (rumenal) ulcers could be produced by ligating the pylorus of a fasting rat, numerous studies have appeared, using this technic, on the effect of various agents on formation of such ulcers and on the composition of the gastric juice which accumulates. It has been found that following adrenalectomy, rumenal ulcers do not develop(2,3) and acid and pepsin production by the mucosa is greatly diminished(4,5). Replacement therapy with corticoids, however, results in ulcer formation (3) coincidental with the return of normal gastric secretion(5). In the present communi-

cation, we report the influence of large doses of cortisol (COL) on gastric ulcers using the Shay rat preparation.

Materials and methods. A total of 98 female rats, of an average body weight of 160 g, of the Upjohn strain (originally from Sprague-Dawley) were divided into groups of 8-10 animals. Half of these served as controls (receiving cortisol vehicle), whereas the other half were treated with cortisol. At 9:00 a.m., 48 hours prior to operation, food was removed. Pylorus ligation was performed after this period of fasting according to Shay's procedure(1); however, in order to avoid any

TABLE I. Effect of Cortisol on Rumenal Ulcers of Animals Killed at Various Times after Pylorus Ligation.

	Sham, 10 hr	3 hr	6 hr	9 hr	10 hr	12 hr
No. of animals/group	10	8	8	8	10	8
No. ulcers*/rat						
CMC	0	0	8.9	11.7	12.9	27
COL	0	0	3.6	5.1	8.3	8.6
Perforations (%)						
CMC	0	0	0	25	20	50
COL	0	0	0	0	10	13
Vol gastric juice (ml)						
CMC		5.3	8.2	10.9	9.2	10.5
COL		4.7	8.3	9.1	9.7	9.8
Pyloric ulcers						
Ulcer index†						
CMC						.0
COL						6.9

* Rumenal ulcers.

† "Ulcer Index" for pylorus portion of stomach is determined by calculating the sum of: incidence in % divided by 10, severity (in pluses) and No. of ulcers/rat. A maximum "ulcer index" is around 18-20.

possible trauma to the gastric mucosa due to introduction of a gastric tube, the stomach was not washed with saline at the time of ligation. Sham operation consisted of manipulating the pylorus without ligating. Water was withheld from the time of operation until sacrifice. COL was given at a dose of 10 mg in suspension in 0.2 ml of CMC (carboxymethylcellulose, Polysorbate 80, propylparaben and water) subcutaneously, once daily, starting at beginning of fasting period (total of 3 injections). Groups of animals were killed at various times after pylorus ligation (3, 6, 9, 10 and 12 hours) (Table I). At autopsy, the stomach was removed, opened along the lesser curvature for inspection, and the volume of accumulated gastric juice was measured. The rumenal (cardiac) and pyloric portions were carefully examined under a magnifier and the number of ulcers recorded. Their severity was graded according to a scale from 0 to ++++.

Results. Rumenal ulcers. There was no ulcer in the sham operated animals and in those killed 3 hours after pylorus ligation (Table I). In the ligated rats receiving CMC and killed later than 3 hours, the rumen contained more and more ulcers with the progress of time. When the animals were treated with COL, the number of ulcers per animal and

their severity were markedly decreased as compared with their respective controls (Fig. 1, 2). Moreover, many untreated animals had gastric perforations whereas the percentage was very small in COL-treated animals.

Pyloric Ulcers. Table I also indicates that the combination of fasting plus treatment with COL promoted formation of pyloric ulcers, i.e., of the corpus of the stomach, in sev-

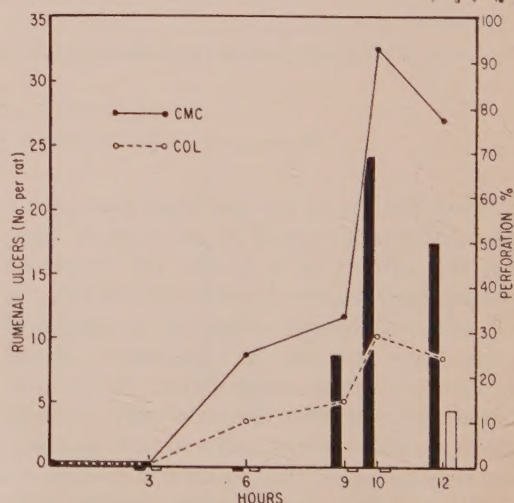


FIG. 1. No. of ulcers/animal (curves) and percentage of perforation (columns) after treatment with COL, in function of time. Black columns, controls; white columns, COL-treated animals.

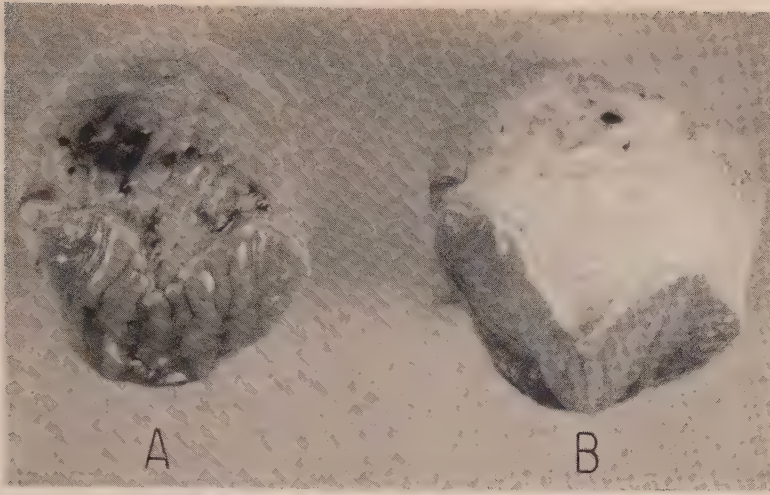


FIG. 2. Effect of COL on ulcers obtained by pylorus ligation. A. Rumenal ulcers of an untreated animal. Numerous and severe. One can see the edema of the rumen. B. Rumen of a COL-treated animal. Only a few ulcers, of moderate severity. No visible edema.

eral animals, giving, for instance, an "ulcer index" of 6.9 in the 12-hour group (see Table I for determination of "ulcer index").

Volume of gastric juice. Cortisol did not seem to influence the rate of gastric secretion following pylorus ligation.

Discussion. Cortisol markedly protected the animals against ulcerations of the rumen which are seen after pyloric ligation to the extent that perforations were rare. The mechanism of this protection is not clear, but it seems highly improbable that COL altered the composition of the gastric juice to render it less irritating to the rumenal epithelium, because of the following observations: (a) it has been shown that glucocorticoids increase peptic activity and acidity of gastric juice both in adrenalectomized rats(3,5) and in man(6); (b) urinary excretion of uropepsin in man is increased by these same hormones (6); (c) in the present experiment, the pyloric portion was ulcerated only in the groups receiving COL.

On the other hand, the protective effect of COL on the rumen might be due to its antiphlogistic property. When one examines stomachs at various times after pyloric ligation, it is striking that the first change to appear in the rumen is gross edema; histologically the edematous fluid is infiltrated with polymorphonuclears and lymphocytes, characteristic of the early stage of an inflammatory

reaction. Later, necrotizing ulcers develop and the reaction becomes more severe. These morphological changes are likely to be brought about by the presence of increasing amounts of gastric juice, the latter being more irritating to the tissues than normally since there is no food to buffer its digestive activity. Therefore, it is understandable that because of its antiphlogistic property, COL blocks this inflammatory reaction (Fig. 3) and hence prevents to a large extent the formation of ulcers in the rumen. Remouchamps(7), using the Shay rat, did not observe any reduction in the number of ulcers with either ACTH or cortisone; this can be explained by the fact that he admittedly used very small doses of hormone.

The presence of pyloric ulcers following treatment with COL is reminiscent of the clinical observations that treatment with glucocorticoids is sometimes followed by gastric distress and even reactivation of ulcers. The present experiment demonstrates a paradoxical effect of COL, namely, inhibition of rumenal and development of pyloric ulcers. This strongly suggests a different mechanism for both types of ulceration. Although the Shay rat can be used with profit for the study of gastric juice under various conditions, the ulcerations obtained with this technic may not be comparable to peptic ulcers; the results of the present experiment indicate that the ru-

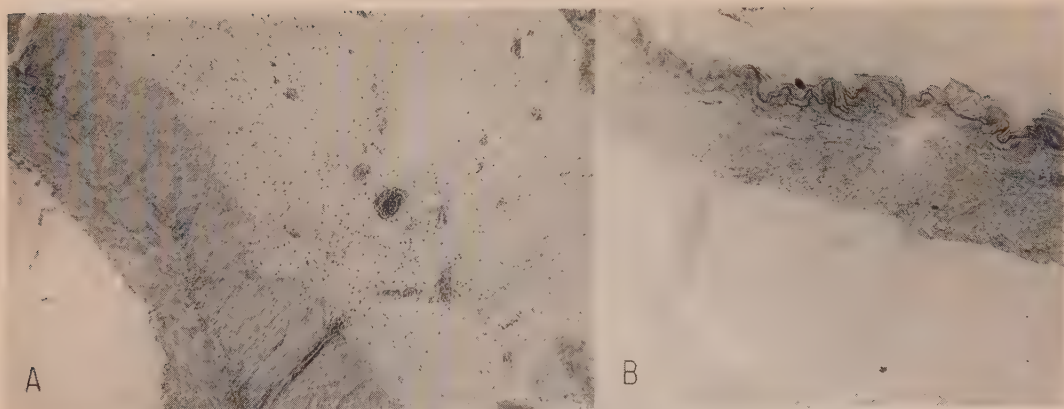


FIG. 3. Effect of COL on rumen of pylorus ligated animals. A. Rumen of untreated rat; tissues are distended by abundant edema and show marked inflammatory cell infiltration. 42 $\times$. B. Rumen of animal treated with COL. No edema, no inflammatory reaction. 42 $\times$.

men, a nonglandular structure, reacts differently than the corpus, which is the secretory portion of the stomach.

The production in the rat of ulcers in the pyloric portion following COL treatment and their expression in terms of "ulcer index" constitute the basis of an ulcer assay for steroids and will be the subject of a separate publication.

Summary. 1) Cortisol (COL) given at the daily dose of 10 mg inhibited to a large extent the development of rumenal ulcers in pylorus ligated rats. It also reduced markedly the incidence of gastric perforation. It is suggested that this protection is due to the antiphlogistic property of COL by which this hormone counteracts the initial inflammatory reaction in the rumen wall brought about normally by concentrated gastric juice. 2) At the same

time, COL promoted formation of ulcers in the corpus of the stomach, whether or not the pylorus was ligated.

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Absorption of Free and Combined Fatty Acids.* (23924)

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Much evidence has accumulated to support two hypotheses that either fat must be either completely or only partially hydrolyzed to permit intestinal absorption(1). Recently,

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the latter of the two possibilities appears to have been more generally accepted. The reported rapid and selective absorption of labeled free fatty acids, when fed in small amounts mixed with a triglyceride(2), would make possible a transfer of mainly fatty acids across the intestinal cell wall as long as even

small amounts of free fatty acids were present for absorption. Also some evidence has been presented to indicate that fed TG are absorbed to a greater extent as free FA than as glycerides(3).

To gain further insight into the major form involved in the absorption of fatty acids, an attempt has been made to follow rates of intestinal absorption of various glycerides and free fatty acids, and the effect upon absorption of their state in the intestinal tract. The finding by Mattson *et al.*(4) of appreciable amounts of the mono-, di-, and triglycerides as well as free fatty acids in the intestinal tract during fat absorption prompted the determination of the rate of absorption of each of these when fed separately. The state of emulsification and degree of hydrolysis of the intestinal lipids have been given such important roles in the absorptive process(5) that these two factors have been reexamined along with the effect of fat supplements which promote lipolysis.

Experimental. Male white rats, weighing approximately 325 g, were fasted overnight and administered a lipid by tube. Either a triglyceride (TG) as olive oil, glycerol dioleate (DG)[†], glycerol monooleate (MG), or free fatty acids (FA) prepared from olive oil, were given in amounts equivalent to the fatty acids in 0.3 ml of olive oil per sq dm of body surface. A similar amount of a mixture of 2 parts TG and 1 part of MG and FA, equal to that formed by the hydrolysis of 1 part of TG, was given in like manner. Blood samples were collected at intervals for chylomicron counts. The experiment was repeated with only 1/3 of the above intake of TG or FA. The areas under the curves obtained were used as relative measure of the rate of lipid absorption(6). In another experiment after a 48-hour fast, rats were administered either olive oil, or fatty acids prepared from it. The animals were sacrificed after 3 hours and separate recoveries were made of the unabsorbed lipid from the stomach and intestines, permitting calculations of relative rates of absorption(7) based either upon total ingested lipid or upon that presented for absorption.

[†] The mono- and dioleate were generously supplied by the Distillation Products Industries.

In a study of the effect of several supplements on fat absorption, 19 rats were placed in 5 groups. All rats were fasted and given 0.15 ml of olive oil per sq dm of body surface, and the intestinal contents were recovered 2 hours later by flushing the intestines with water. The washings were made up to uniform volumes and the optical density of each was determined as a measure of the state of emulsification of the fat present, according to the method of Mead *et al.*(8). The contained lipids were recovered and titrated with alkali to determine the extent of lipolysis of the recovered lipids. Recovery of fat from only the intestines again permitted the calculation of fat absorption on the basis of the lipid which had passed out of the stomach and was readily available for absorption. In the last part of this investigation, the rates of appearance of particulate fat in the blood were followed after giving 0.3 ml of olive oil, as in the first experiment, along with a single supplement of 2 ml of 0.1 M solutions of histidine, ethionine or glycine. The method of Balls *et al.*(9) was used to determine the effect of the supplements used on the rate of fat hydrolysis.

Results. Rates of absorption of ingested TG and DG were similar when measured by the increase of particulate blood lipid (Table I). No increase in the rate of absorption was obtained after administration of a like amount of a mixture of TG, MG, and FA previously reported(5) to best promote emulsification

TABLE I. Relative Rates of Appearance of Particulate Blood Lipid after Feeding Glycerides or Fatty Acids.

No. rats	Lipid given*	Amt fed, ml/dm ²	Area under chylo. curvet 1-3 hr	"t" values
10	TG	.30	99 ± 4	
10	DG	.32	92 ± 2	1.53
10	MG	.34	55 ± 3	9.08
13	FA	.30	70 ± 3	6.11
10	TG + MG + FA	.30	94 ± 2	.91
19	TG	.10	70 ± 2	
19	FA	.10	73 ± 1	1.40

* Tri-, di- and monoglycerides are designated by TG, DG, and MG respectively. The mixture contains 2 parts of TG and 1 part of its fatty acid (FA) equivalent of MG and FA.

[†] Stand. errors of mean and "t" values are included. All "t" values greater than 3 are statistically significant.

TABLE II. Absorption of Lipid Fed as Free and Combined Fatty Acids.

No. of rats	Lipid fed		Lipid recovered after 3 hr		Lipid absorbed	
	As	Amt, ml/dm ²	Stomach	Intestine	Based on total fed	Based on avail. fat*
					%	
9	Olive oil	.1	11	23	66	74 ± 3
8	Oleic acid	.1	50	15	35	70 ± 4
12	Olive oil	.3	34	26	40	61 ± 4
9	Oleic acid	.3	62	14	24	63 ± 7

* Available fat is total ingested less that retained in the stomach. Stand. error of mean is included.

and absorption. The lower blood fat levels after ingestion at this level of intake of MG or free FA were the result of gastric retention (Table II). However, similar blood fat levels were obtained by this method after very low intakes of either FA or TG. Both MG(10) and FA at both levels of intake disappear from the intestinal tract as rapidly as TG if only lipid that has passed from the stomach is considered (Table II).

A comparison of the state of emulsification or degree of hydrolysis of intestinal fat with the per cent of lipid absorbed was made by obtaining the ratio of either optical density (D) or the per cent hydrolysis (H) to the percentage of available fat absorbed (Table III). Variations in the ratios in the latter are much smaller (Table III). To the extent that such variations may be used as a measure of a relationship, the hydrolysis of the fat in the intestine appears to be more related to the rate of absorption than to the state of emulsification of the lipid.

A markedly accelerated rate of *in vitro* hydrolysis of olive oil by lipase in the presence

of histidine has been reported by Yamamoto (11). Similar results were obtained with histidine and ethionine but not with glycine. As measured by increases in particulate blood fat, only the fat supplements which were found to increase the speed of fat hydrolysis were associated with significant increases in the rates of fat absorption (Table IV).

Discussion. Although most data from labeling technics have been interpreted as showing that not more than 50% of the TG is completely hydrolyzed before absorption, only traces of TG have been found to be absorbed unchanged (12,13). If this is true, a complete hydrolysis of half of the TG would necessarily be associated with a partial hydrolysis of most of the remaining TG and necessitate the absorption of most of the total FA as free FA.

The reported slower absorption of free FA than TG (2,14) can be attributed at least in part to a slower passage of FA from the stomach when more than very small amounts are ingested. The slightly greater amounts of FA recovered from the intestine after feeding an excess of FA with a fat was said by Borg-

TABLE III. Comparison of Variabilities in Ratios of Optical Densities or Degree of Hydrolysis of Fat in the Intestine with Fat Absorption.

Group No.	No. of animals	Intest. contents		Available fat* absorbed (A)	D†/A	H/A
		Optical density (D)	Fat hydroly. (H)			
I	4	.465	43	73	.64	.59
II	4	.481	43	64	.75	.67
III	4	.683	38	57	1.20	.67
IV	3	.371	45	70	.53	.64
V	4	.590	55	80	.74	.69
Avg ± stand. dev.					.77 ± .25	.65 ± .04
Stand. dev. × 100/av					39	6

* Absorption values based on recoveries of available fat—total fed less that retained in the stomach.

† Optical density values × 100.

TABLE IV. Effect of Histidine, Ethionine and Glycine Supplements on Fat Hydrolysis and Absorption.

No. rats	Fat supplement		Area under chylo. curve 1-3 hr†	“t” value
	As	Hydrolysis ratio*		
9	None	1.0	94 ± 4	
10	Histidine	2.5	136 ± 3	3.38
10	Ethionine	2.0	141 ± 6	7.98
7	Glycine	1.0	96 ± 6	

* Ratio of hydrolysis of lipid with .02 M supplements as compared with that of none.

† Stand. error of mean included. All “t” values over 3 are significant with a P value of less than .01.

ström(2) and Mattson *et al.*(4) to indicate a limited absorption of FA. In this study, the smaller amounts of lipid found in the intestine after feeding only FA, as compared with that after TG ingestion (Table II), suggests that FA are readily absorbed when available for absorption. Undoubtedly, small amounts of free FA when fed as such are absorbed at least as rapidly as the FA of the readily hydrolyzable TG and DG (Tables I, II).

The constancy of the contents of the lumen, almost regardless of the form in which the fatty acid is ingested(4), certainly cannot be accepted as proof of the absorption in any one form. It could well be a means of preventing any unphysiological effect of excessive amounts of free FA in the intestine. Normally, instead of flooding the gastrointestinal tract with large quantities of free acid, a small amount of FA is slowly released by a continuing hydrolysis and constantly removed by absorption. A selective absorption(2) of any FA present could promote the transfer of most of the lipid in that form.

The importance of the role of FA in the absorptive process is suggested by 4 findings in this study. (1) The free FA appear to be at least as rapidly absorbed as are those of TG (Table II) if only the amounts actually available for absorption are considered. (2) The percentage of FA in the intestines bears a much less variable relationship to rate of absorption than degree of emulsification (Table III). (3) An attempt to promote emulsification by the presence of an ideal mixture of fat with MG and FA, according to Frazer(5), did not hasten the absorptive rate

(Table I). (4) Furthermore, fat supplements which accelerate the action of the lipolytic enzyme are associated with marked increases in rate of absorption of the ingested fat (Table IV). This finding supports the suggestion(15) that a direct relationship exists between rate of hydrolysis and speed of absorption.

Indisputable evidence is not available regarding the exact quantities of the various forms in which the fatty acids are absorbed. Results of this study are in agreement with the suggestion that the major part of the fat absorbed from the intestinal lumen may be in the form of the unesterified FA.

Summary. Evidence is presented which suggests that free FA are at least as rapidly absorbed as those of the readily hydrolyzable glycerides when the rate is measured either by increased blood fat levels or by disappearance of available lipid from the intestinal tract. The absorptive rate appears to be more related to the degree of hydrolysis of the intestinal lipid than to its state of emulsification. Conditions which promote lipolysis increase the rate of fat absorption. These findings support the hypothesis that the major part of the fat absorbed probably is in the form of free FA.

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Cerebrospinal Fluid Electrolytes after Death. (23925)

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While isolated data on electrolytes in postmortem cerebrospinal fluid have been reported (1,3,5), a systematic study of the principal ions has not been available. The present report gives analyses of 157 postmortem cerebrospinal fluids and includes values for cations, anions and pH.

Materials and methods. The material consisted of 131 male bodies, average 57 years old, of white and colored race, autopsied on the average $10\frac{1}{2}$ hours after death. A second series was used for pH determinations only and consisted of 26 cases, 62 years average age, examined after an average $5\frac{1}{2}$ hour postmortem interval. To obtain data, which may be valid as "normals," cases were selected on the basis of pathological changes presumably unrelated to electrolyte metabolism. Cerebrospinal fluid was removed by cisternal puncture and, if free of gross contamination by blood or brain tissue, was used after centrifugation. The following analytical procedures were employed: flame photometry for sodium and potassium(4); Clark-Collip's method for calcium(2); Denis-Briggs' method for magnesium(2); Van Slyke-Hiller's modification of Sendroy's method for chlorides(2). The CO_2 values were previously published(5) and obtained by Van Slyke's gas volumetric method(2). Measurements of pH were performed at 37.5°C (within 60 min. after removal) with a Beckman pH meter and a blood electrode assembly provided with rubber cap, through which the spinal fluid was injected directly from syringe or after centrifugation under oil.

Results. Average and range of cations, anions and pH in postmortem cerebrospinal fluid $10\frac{1}{2}$ and $5\frac{1}{2}$ hours after death respec-

tively are compiled in Table I and compared to corresponding antemortem values. It will be noted that there is a marked increase of potassium and inorganic phosphorus, a moderate increase of magnesium and a slight to moderate decrease of sodium, chlorides and HCO_3 after death, while calcium values approach closely those found during life. There is, however, more or less marked scattering of all values over wide ranges. The postmortem values of pH are considerably lower than antemortem figures.

The acid-base equilibrium in postmortem cerebrospinal fluid as seen in Table II shows fair agreement of the total milliequivalents of cations with the antemortem figure due to the fact that the sodium deficit is compensated by the potassium increase. On the other hand, there is a large deficit of 24 milliequivalents of anions due to the decrease of chlorides and HCO_3 ions in postmortem cerebrospinal fluid and insufficient compensation by the increase of phosphorus. This result causes an apparent shift of the acid-base equilibrium to the side of base.

Discussion. Mason, Klyne and Lennox(3) first observed the marked postmortem rise of cerebrospinal potassium, which however, failed to show close correlation with time after death except on a statistical basis. These findings were borne out by the present investigation, and here again a constant time relation was absent in regard to postmortem changes of sodium, chlorides and phosphorus. The increase of potassium and the decrease of sodium and chlorides are obviously due to autolytic changes of cell membranes with resultant greater permeability, so that intra-

TABLE I. Postmortem and Comparative Antemortem Values of Cerebrospinal Fluid Electrolytes (131 Cases) and pH (26 Cases).*

Electrolytes		Postmortem values			Antemortem values				Diff. in % of antemortem Avg
		Avg	SD, % of avg	Range	Avg	Range			
Na	mg %	292		247 - 344	328	308	-352 (7)		- 11
	meq/l	127	3.2	107 - 150	143	134	-152 (7)		
K	mg %	82		22 - 157	11.8	9.8	- 14.3(7)		+610
	meq/l	21	6.1	5.6 - 40.0	2.9	2.5	- 3.7(7)		
Ca	mg %	4.8		3.8 - 6.8	5.0	4.5	- 5.2(8)		- 4
	meq/l	2.4	6.2	1.9 - 3.4	2.5	2.3	- 2.6(8)		
Mg	mg %	3.5		2.3 - 4.4	2.4	2.0	- 2.6(8)		+ 45
	meq/l	2.9	8.6	1.9 - 3.7	2.0	1.7	- 2.2(8)		
CO ₂	vol %	21		10 - 58	59	57	- 62 (8)		- 64
HCO ₃	meq/l	9.4	3.3	4.5 - 26	26	25	- 28 (8,11)		
Cl as NaCl as Cl " "	mg %	663		519 - 833	730	697	-748 (10)		- 10
		402		315 - 505	444	423	-454 (10)		
	meq/l	113	2.2	89 - 142	125	119	-128 (10)		
P (inorg.)	mg %	8.9		1.2 - 18.0	1.4	.9	- 2.0(6)		+550
	meq/l	5.2	5.6	.7 - 10.5	.8	.5	- 1.2(6,11)		
pH		6.38	.2	6.03- 6.95	—	7.35-	7.44(10)		

* For specified conditions see text.

cellular ions of higher concentration such as potassium may diffuse out into the extracellular fluid, and—vice versa—extracellular ions of higher concentration such as sodium and chlorides may diffuse into the cells. The increase of phosphorus, on the other hand, may be interpreted as evidence of postmortem esterase activity.

The stability of calcium in postmortem serum was noted by Hartl, Waldmann and Eder(1) and was presently confirmed also in postmortem cerebrospinal fluid. The moderate increase of magnesium may be explained similarly as that of potassium by diffusion from the cells, which contain a higher concentration of magnesium than extracellular fluid.

The apparent shift of the acid-base equi-

lium to the side of base is in contrast with the actual acidity of postmortem cerebrospinal fluid as evidenced by the lowered pH values, which were observed recently also in postmortem blood by Straumfjord and Butler(9). This postmortem acidity is most likely caused by liberation of organic acid radicals, *e.g.* lactic acid, which presumably overcompensate the apparent shift to alkalinity.

Summary. (1) Cations and anions of postmortem cerebrospinal fluid have been studied in 131 cases, and pH determinations performed in an additional 26 cases. (2) Postmortem changes observed include marked increases of potassium and inorganic phosphorus, moderate increases of magnesium, slight to moderate decreases of sodium, chlorides and HCO₃ and a relative stability of calcium ions, the concentration of which approached closely antemortem values. (3) The acid-base equilibrium in postmortem cerebrospinal fluid was found shifted to the alkaline side by a deficit of anions in contrast to the actual acidity as determined by pH measurements, presumably due to liberation of organic acid radicals. (4) Postmortem electrolyte changes may be interpreted by greater permeability of cell membranes due to autolysis, while the increased inorganic phosphorus may result from postmortem esterase activity.

TABLE II. Postmortem and Comparative Antemortem Acid-Base Equilibrium in Cerebrospinal Fluid (131 Cases).

Cations			Anions		
	Post-mortem meq/l	Antemortem meq/l		Post-mortem meq/l	Antemortem meq/l
Na	127	143	Cl	113	125
K	21	2.9	HCO ₃	9.4	26
Ca	2.4	2.5	P	5.2	.8
Mg	2.9	2.0	S	1 *	1
Totals	153.3	150.4		128.6	152.8

* Normal antemortem value assumed in postmortem fluid.

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Growth and CF Antigenicity of Measles Virus in Cells Deriving from Human Heart. (23926)

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Studies on virus susceptibility of cells deriving from adult human heart tissue indicated that measles virus attained comparatively high titers in such cultures(1). Availability of these preparations and the relative paucity of data on antigenicity of inactivated measles virus led us to employ complement-fixation (CF) as a method of antigenic investigation. The contributions of Enders and his group have included data on ability of tissue culture grown measles virus to serve as diagnostic CF antigen(2). Ruckle and Rogers(3) extended this approach to more fully characterize the antibody response. The material which follows amplifies these basic data.

Methods. A. Tissue Culture: Source of original tissue was a biopsy specimen from right atrial appendage of human heart removed during commisurotomy. Cultures were prepared by classic chick plasma clot method using minced tissue and basal medium (Eagle) containing 20% human serum. Growth of a fibrocytic type cell was observed after about 72 hours and became progressively extensive. Serial transfers to daughter cultures were performed after trypsinization of the sheet. After 4 months, the predominant cell type had become epithelial-like and has since propagated as such. *Tube cultures*, prepared by seeding about 70,000 cells/tube in 0.5 to 1 ml of above medium, were ready for

use at 24 to 48 hours. Washed cultures were then maintained on medium containing 2 to 5% of either horse or chicken serum, 65% Scherer's MS and 30% Medium 199. The balanced salt solution portions of each of the synthetic media were according to the formula of Earle. *Virus.* Edmonston strain of measles virus was obtained from Dr. M. Reissig and had been through 23 human kidney passages, one HeLa cell passage and 4 passages in the Hep-2 cell. Production of initial pools of virus was performed by inoculation of tube cultures of heart cells. When cytopathic effects indicated that almost all cells had been infected (3 to 4 + degeneration), the cultures were frozen and thawed and supernatant fluid of lightly centrifuged preparations (TCF) re-inoculated into fresh cultures. *Titration*s were performed by inoculating 2 to 4 culture tubes each with 0.1 ml of given virus dilution prepared in Hanks' BSS, and observing them for cytopathic effects daily for 14 days. Degeneration of fusiform or stellate type at dilution endpoint usually began by 8th day and progressed to completion by 12th to 14th day. *Complement-Fixation.* Crude tissue culture fluid antigens were prepared by inoculating flask cultures containing about 30,000,000 cells with large virus inocula (1 to 10 x 10⁶ TCD₅₀). The basal medium in this instance was supplemented with 2% chicken serum.

When complete cell degeneration had occurred, the cultures were frozen at -20°C and following thawing and light centrifugation the supernatant fluid was harvested as crude antigen. Concentrated antigens were prepared by ultracentrifuging crude infected fluids to which was added gelatin (final concentration 0.06%) (4) for 90 minutes at 30,000 rpm in Spinco #30 rotor (78,000G). Upon completion of the run, the supernatant fluids were decanted and the pellets resuspended to 1/10th original volume in Hanks' BSS. The CF method was based on the Kolmer procedure and employed overnight fixation at $4-6^{\circ}\text{C}$, and 30 minute incubation at 37°C for the hemolytic system. Tests were performed in tubes employing 0.25 ml each of antigen and serum dilutions and 0.5 ml of complement previously titrated and adjusted to contain 2 full units. Hemolysis was read visually and 2 plus or greater fixation was considered as positive. A control antigen, prepared from non-infected human heart tissue culture, was included in each test. *Preparation of Antisera.* Guinea pigs were inoculated at 3 weekly intervals with 1 ml of infectious measles virus prepared in *monkey kidney tissue cultures*. After 7-10 days, they were bled by cardiac puncture. Such sera, used for control purposes only, did not contain CF antibodies against human heart tissue or chicken serum, both component parts of the CF antigens employed in these studies. Monkey immune serum from a measles convalescent animal was kindly furnished by Dr. J. Melnick.

Results. Optimal harvesting time for infectivity and CF activity. Table I indicates results of 2 experiments to show appearance of infectivity and complement-fixing activity in crude culture fluids. These properties appeared highest at 4 or 5 days after infection, and although further incubation seemed to decrease infectivity, the CF titer remained unchanged when tested against an immune monkey serum. *Effect of inactivation methods on CF antigenicity.* Because a non-infectious antigen would be desirable as a diagnostic reagent for measles, 3 methods of inactivation were investigated for their relative ability to maintain CF activity while rendering the preparation non-infective. (a) *Heat inacti-*

TABLE I. Relation of Harvesting Time to Yield of Measles Virus.

Day of harvest	Cytopathic level*	T.C. infectious titer/ml	CF titer
3	1	4.8	<1:2
4	2-3	6.8	"
5	3-4	"	1:2
6	4	"	"
7		"	"
3	1-2	5.8	1:2
4	3-4	7.8	1:4
5	4	6.8	"
6		"	"
7		5.8	"
10		"	"

* 1 = 25% degeneration; 4 = complete degeneration of culture.

vation was performed by immersing infected fluids in a 56°C water bath and replicate sealed tubes were removed at various times for infectivity and CF tests. (b) *Formalin inactivation.* One part of a 1:400 dilution of formaldehyde, U.S.P. was added dropwise to 9 parts of an infectious measles virus preparation held in ice bath with constant agitation (final concentration 1:4000). The preparation was then incubated at 37°C and sampled for assay at various times. (c) *Ultraviolet light.* Clarified infectious culture fluids were irradiated with the Habel-Sockrider apparatus (5) employing a flow rate of about 7 ml/minute. The irradiation cycles were repeated several times, a sample being removed for assay after each cycle. Tests for inactivation were performed as follows. A 10 ml sample was inoculated into 10 ml of culture medium in 24-36 hour old flask cultures containing about 5×10^6 cells. These were incubated at 37°C for one week at which time 10 ml of the fluid was passed into fresh cultures and old cultures refed. Both sets were re-incubated for an additional week and passages repeated 3 times. In all cases where live virus was found, the cytopathic effect was obvious in the initial passage; if doubtful initially, it was noted on first passage into new flasks. It was also of interest to note that if live virus was detected by these rather exhaustive procedures, it was also seen in titration tube cultures receiving undiluted inoculum. Table II presents results of 2 experiments employing inactivation methods described above. Infectivity had en-

TABLE II. Inactivation of Measles Virus by Heat, Formaldehyde or Ultraviolet Light.

Procedure	Exp.	Time	Infectious titer	CF titer		
Min.						
Heating at 56°C	1	0	7.8	1:4		
		30	3.8	<1:4		
		75	<Und.	ND		
		120	"	"		
		150	"	<1:4		
	2	0	5.8	1:4		
		30	1.8	ND		
		45	.8	1:2		
		60	<Und.	"		
		75	"	"		
Days						
Formaldehyde, 1:4000 at 37°C	1	0	6.8	1:8		
		3	2.8	"		
		5	<Und.	"		
		7,9,11,13	"	"		
		2	0	4.8	1:4	
	2	3	.8	"		
		4	<Und.	"		
		5,6,7	"	"		
		Exposure cycle				
		Ultraviolet ir- radiation	1	0	6.8	1:8/ 1:8*
1	1.8			" / 1:4		
2	<Und.			1:4/<1:2		
3	"			"		
2	0			6.8	1:4	
2	1		.8	1:2		
	2		<Und.	<1:2		

* *vs* monkey hyperimmune serum/*vs* human convalescent serum.

ND = not done; Und. = undiluted.

tirely disappeared after 60 minutes at 56°C, after 4 days with 1:4000 formaldehyde at 37°C, and after 2 cycles of ultraviolet irradiation. The CF titer of the TCF was reduced by heat or ultraviolet irradiation but was not affected by formaldehyde even when held at

37°C for a 13-day period.

Stability of frozen-dried measles CF antigen. Because of its potential usefulness in epidemiologic studies of measles infection, the stability of measles CF antigen following freeze drying was determined. Five ml of infected culture supernatants were shell-frozen and lyophilized for 20-22 hours on an Aminco dryer. No external heating was employed. At completion of the run, the ampules were filled with "super-dry" nitrogen and flame sealed. Such preparations retain some infectivity and exhibit considerable CF stability. A comparison of CF antigen content in wet and dried culture fluids stored under various conditions is shown in Table III. After 14 days in the cold or at room temperature there was no loss of CF antigenicity and even after exposure to 37°C for this interval there was considerable residual CF activity. Although longer intervals have not been investigated, these results suggest that measles virus can be stored in the dried state at low temperatures for considerable periods and retain its usefulness as a CF antigen. In contrast, preliminary evidence has indicated that the original titer ($10^{-6.8}$ /ml) of wet material does not decrease in 21 days at either 4°C, -20°C, or -70°C. However, after 18 weeks at -70°C, there was considerable loss of titer (10-4).

Effect of sonic vibration on measles virus. Exposure of measles virus in crude tissue culture fluids to sonic oscillation (10 kc/sec at 4-6°C in a Raytheon oscillator driven at 1.25 amps.) resulted in complete loss of infectivity after 2 hours. This was accompanied

TABLE III. Effect of Freeze-Drying on Infectivity and CF Antigenicity of Measles Virus.

Treatment	Exp.	Storage		TCD ₅₀ /ml	CF titer <i>vs</i> 4 units	
					Rhesus immune serum	Human convalescent serum
None—Crude TCF	1	5°C		6.5	1:16	1:4
Freeze-dried		5°C	14 days	—*	"	"
		23-25°C	14 "	—	"	—
		37°C	14 "	—	1:8	1:4
None—Crude TCF	2	5°C		4.8	1:4	"
Freeze-dried		"	7 "	2.0	"	"
None—Crude TCF	3	5°C		5.8	1:8	"
Freeze-dried		"	29 "	—	"	—

* — = not done.

by partial loss of CF activity which became evident after 1 hour. It is of interest that a similar exposure did not diminish infectivity of poliovirus or several types of ECHO viruses.

CF reaction between measles antigen and human and animal sera. a. *Concentration of CF antigen:* In the procedure described, supernatant fluid from measles infected human heart cell cultures will fix complement with hyperimmune monkey sera to maximal dilutions of 1:4-1:8. For early diagnosis of measles, where antibody levels may be low, at least 2 full antigen units are desirable. To achieve this consistently, crude infectious culture fluids have been concentrated 10 times by ultracentrifugation. This results in increased serum titers with hyperimmune monkey or convalescent human sera. Two grid

TABLE IV. CF Reactions of Crude and Concentrated Measles Virus with Immune Human and Monkey Sera.

Serum		Infectious antigen dilution end-point	
Type	Dilution	Crude TCF	Cone., 10X
Human acute pool	1: 16	1:2	1: 2
Human convalescent pool	1: 16	1:4	1: 8
	1: 32	"	"
	1: 64	1:2	1: 4
	1:128	"	"
Rhesus immune	1: 16	1:4	1:32
	1: 32	"	1:16
	1: 64	"	"
	1:128	"	"
	1:256	"	1: 8
	1:512	"	"

titrations illustrating this are found in Table IV. In the first experiment a pool of acute (less than 4 days after appearance of illness) and convalescent (14 days or more) human sera* were tested against crude and 10X concentrated infectious antigens. No fixation occurred with acute phase sera at a dilution of 1:16. The convalescent pool had a titer of 1:128 against concentrated and 1:32 against crude antigens. A 4-fold increase in antigen titer, from 1:4 to 1:16, was also observed. Tests of a potent convalescent serum obtained

*We wish to express our thanks to Dr. Robert Ward for providing these sera.

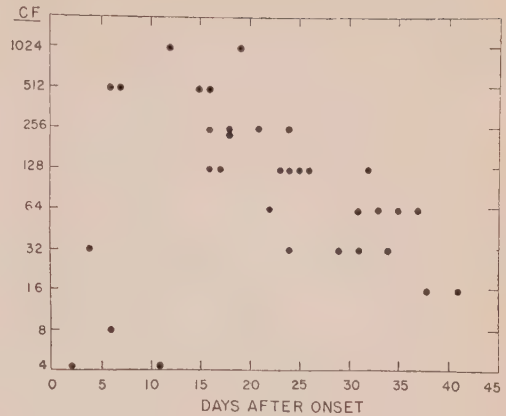


FIG. 1. Distribution of measles CF antibody titers in 33 cases of clinical measles.

from a rhesus monkey following inoculation of Edmonston strain of virus provided additional example of increased CF reactivity which accompanies concentration of antigen.

b. *Development of measles CF antibody in man:* A series of human sera was obtained from patients with clinical measles, from healthy adults with a history of childhood measles, and from a small series of adults who denied having the disease. The CF antibody levels in these sera were determined with concentrated measles antigens. In general our findings paralleled those obtained by Enders *et al.*(2) and Ruckle and Rogers(3) who employed crude tissue culture fluid antigens. Time of appearance of CF antibody in a group of 33 children with clinical measles is shown in Fig. 1 and exemplified in data from serial bleedings on one 6-year-old child (Fig. 2). In practically all of these cases, the interval is based from time of appearance of rash; in 3 instances from onset of fever; in 3 other cases the onset symptoms are not stated. Sera of 15 individuals obtained prior to onset of

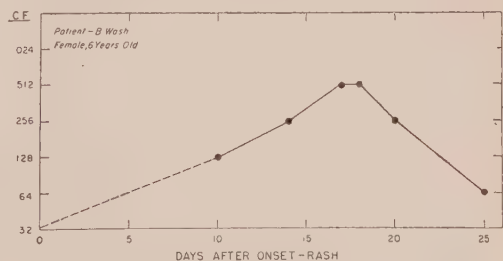


FIG. 2. Development of measles CF antibody following clinical measles.

TABLE V. Persistence of CF Antibody in Adults with History of Childhood Measles, in 14 Sera.

Age, yr	Measles CF antibody	
	With crude TCF*	With conc. virus*
44	1: 4	1: 16
41	1: 8	1: 64
31	1: 4	1: 16
30	1:32	1: 32
30	1: 4	"
28	"	1: 64
28	"	1: 16
27	"	1: 8
27	<1: 4	"
25	1: 8	1: 32
25	1:32	1:256
22	1:16	1:128
?	1: 4	1: 32
?	1: 8	"

* Infectious antigens used.

measles did not react at a dilution of 1:8. Ruckle and Rogers(3) have demonstrated that there is a close correlation between levels of measles neutralizing and CF antibody in man and therefore we have not measured the former antibody in these sera. On the basis of data shown here, it is apparent that appearance of measles CF antibody to readily detectable levels is rapid and prolonged. To establish whether concentrated measles antigens were more sensitive for detection of residual CF antibody, the sera of 14 adults with a history of measles in childhood were collected and titrated against crude and concentrated virus. As will be observed in Table V, low CF levels (1:4-1:32) were obtained using crude TCF; a higher titer was almost invariably noted when 10X concentrated measles antigen was employed. All sera failed to react with a control of non-infected human heart TCF. This demonstration of persistence of measles antibody in adults is more striking when one considers the CF levels in children and adults who have no history of this disease. No serum from 15 children under 6 years of age and 3 adults had a CF titer in excess of 1:8. This suggests that CF antibody determination may provide a simple method for estimating either prevalence of measles in a population group or immunity in an individual.

Discussion. Successful propagation of measles in tissue culture by Enders *et al.* has enabled the application of many conventional

technics of virology to the study of this disease. The human heart cell employed in our studies, like the Hep-2 cell as reported by Black, Reissig and Melnick(6), provides a relatively large yield of virus and is capable of propagation in stable serial passage. Benyesh, *et al.*,(7) using ionizing radiation and gradocol membrane filtration have shown that measles virus has a particle size of 100-200 m μ whereas the size of the CF antigen based on irradiation sensitivity is considerably smaller. The resistance of CF antigen to inactivation after infectivity is lost may well be associated with this size differential.

The CF antibody in measles, unlike that in most viral diseases, appears to develop and persist with the same chronology as the neutralizing antibody(3). Since persons without a history of measles do not have CF antibody and since it makes its appearance shortly after onset of illness and rapidly reaches a high titer, the detection of CF antibody provides a simple procedure not only for diagnosis, but also for establishing the immune status of an individual or group. Freeze-dried, non-infectious antigen prepared from crude or concentrated tissue culture fluids may constitute a simple, safe and stable reagent for this purpose.

Summary. The infectivity and CF antigenicity of measles virus grown in a serial passage human heart cell culture have been investigated. Several methods of inactivation are described, as well as the use of crude or concentrated CF antigens for the detection of measles antibody.

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Effect of Propylthiouracil on Adrenal-Regeneration Hypertension in the Rat. (23927)

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It is well established that important functional inter-relationships exist between thyroid gland and adrenal cortex. In animals rendered hyperthyroid the adrenal cortex enlarges, and it shrinks in size in animals made hypothyroid by thyroidectomy or antithyroid drugs. Studies in this field have been summarized by Deane and Greep(1). Recently Masson *et al.* showed that treatment of adrenal-regeneration hypertensive rats with thyroxine resulted in higher blood pressures and increased the severity of vascular lesions (2). Skelton reported that thyro-parathyroidectomy prevented development of hypertensive vascular disease in rats following adrenal enucleation(3). In this study we found that treatment of adrenal-regeneration hypertensive rats with propylthiouracil (PTU) caused a marked depressant effect on hypertension. These results are of interest in interpretation of the mechanism of action of Amphenone which has antithyroid properties and also causes a reversal of adrenal-regeneration hypertension in the rat(4,5).

Methods. The animals were male Wistar rats weighing 80-100 g. The operative techniques we used have been described(5). In this study, groups of 10 animals were prepared as follows. Group 1 unilateral nephrectomy, Group 2 unilateral nephrectomy, right unilateral adrenal enucleation and contralateral adrenalectomy. Group 3 was operated upon as described for Group 2 and 5 weeks after operation treatment was begun with PTU and continued for duration of experiment. All animals were fed commercial rat food during experimental period and were given 1% saline to drink after operation. PTU was administered orally at dose of 40 mg/kg daily in water. Systolic blood pressures were measured before operation and 3 times weekly during experiment, by the method of Kersten and Brosene(6). At end of experiment the animals were killed with chloroform, tissues were

weighed fresh on torsion balance and prepared for histological examination. Pituitary glands were stained by the method of Rona and Morvay(7) the remainder of the organs were stained by methods as described earlier(5).

Results. I. *Blood pressure.* Hypertension which develops following unilateral adrenal enucleation and contralateral adrenalectomy in unilaterally nephrectomized rats given 1% sodium chloride to drink is illustrated in Fig. 1. Systolic blood pressure during 5-week period rose from normal levels to a mean pressure of 180 mm of mercury. After this point the pressures reached a plateau and remained at hypertensive levels for duration of experiment. Rats which are unilaterally nephrectomized and receive saline show a moderate rise in blood pressure but fail to develop hypertension (Fig. 2). The effect of PTU on adrenal-regeneration hypertension is shown in Fig. 3. The PTU was administered to these animals when mean blood pressure was 160 mm of mercury and was continued for duration of experiment. During this time the usual hypertensive response failed to develop and blood pressure progressively declined to levels comparable to those of Group 1 controls.

II. *Mean tissue weights* of the groups are shown in Table I. Kidney and heart weights are greatest in the adrenal-regeneration hypertension group. Relationship between tissue weights of this group and that treated with PTU is obscured by failure of animals from the latter group to gain weight in a normal manner. Although absolute mean weights for heart, kidney, spleen, thymus and adrenal gland of PTU treated rats are significantly lower than those of adrenal-regeneration hypertension animals, these differences are, however, not significant when values are related to body weight. Thyroid and pituitary glands of PTU treated animals are significantly larger than those of other groups.

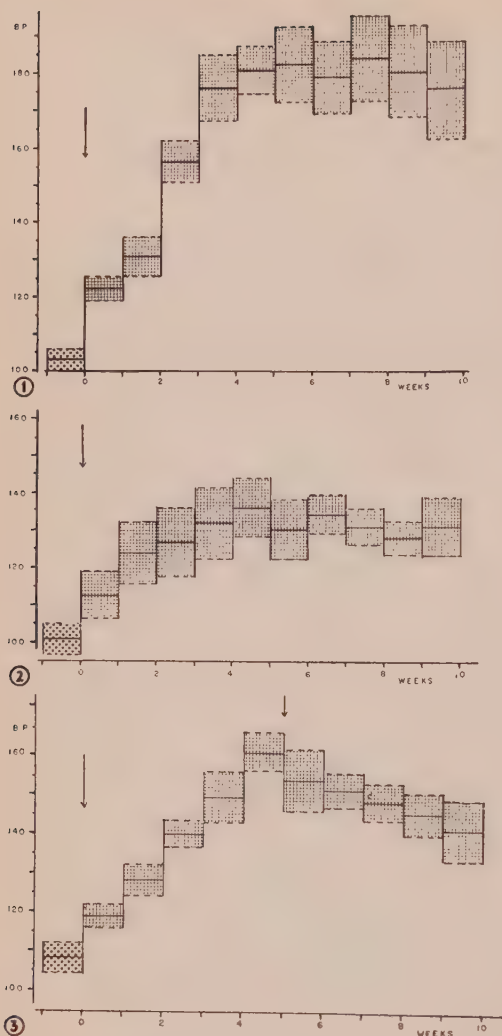


FIG. 1. Hypertensive response in rats of Group 2. Animals were surgically prepared at arrow. Solid horizontal lines represent mean weekly-blood pressure of group and shaded areas are 95% confidence limits.

FIG. 2. Moderate rise in blood pressure after unilateral nephrectomy and saline.

FIG. 3. Depressant effect of PTU treatment on blood pressure of adrenal-regeneration hypertensive rats. Animals were prepared at left arrow and PTU treatment was started as indicated by arrow on right.

III. Histopathological lesions. Group 1. Only one animal in this group showed any evidence of pathological lesions. This rat had a mild degree of chronic focal pyelonephritis without glomerular or vascular involvement. Terminal blood pressure of this animal was 159 mm of mercury.

Group 2. Renal and vascular lesions in this group correspond closely to those we have previously described in animals with adrenal-regeneration hypertension (5). One animal had a particularly severe nephrosclerosis and widespread vascular lesions of brain, thymus, mesentery, intestine, heart and adrenal capsule. Arteriolar necrosis and productive endarteritis in this animal was associated with encephalomalacia in the cortical area with lipid and hemosiderin laden microglial cells surrounding the degenerated areas. Vascular lesions in the heart were found associated with areas of myocardial infarction. Four additional animals from this group had nephrosclerosis and vascular lesions which were less severe. The regenerated adrenal glands showed distinct zonal differentiation and a variable degree of nodularity. The zona glomerulosa was thin and composed of flattened, darkly stained cells with some traces of lipid. The zona fasciculata was well developed and the cells contained a moderate amount of lipid, occasional cell groups were swollen and vacuolated. The zona reticularis was distinct and normal in appearance. In pituitaries of these hypertensive animals a consistent change was noted in the basophils. These cells were increased in number and size compared to animals from Group 1. From 10 to 20% of these basophils contained clearly delineated single or less frequently multiple vacuoles which were approximately equal in size to the nuclei. In these vacuoles some evenly distributed purple granules were seen (Fig. 4). No changes were seen in eosinophil or chromophobe cells. The histological picture in thyroid, pancreas, liver and other organs was not remarkable.

Group 3. No evidence of either renal or vascular lesions was found in animals of this group. The regenerated adrenal gland was small and contained large masses of fibrous connective tissue and calcified areas. Zonation of the regenerated cortex was distinct, though marked differences were apparent compared to the animals of Group 2. The zona glomerulosa was normal in width and composed of darkly stained cells containing little lipid. The zona fasciculata was quite narrow and this difference in size appeared to

TABLE I. Tissue Weights.

	Group 1	Group 2	Group 3
	Unilateral nephrectomy	Adrenal-regenera- tion hypertension	Adrenal-regeneration hypertension + PTU
Thymus (mg)	392 ± 38	333 ± 20	218 ± 15 *
Thyroid	23.7 ± 1.5	25.7 ± 2	61.6 ± 4 *
Adrenal, s	48.6 ± 2.4	34.1 ± 1.2	21.4 ± 1.3 *
Pituitary	11.0 ± .5	12.6 ± .9	16.4 ± .8 *
Spleen	629 ± 30	624 ± 22	444 ± 31 *
Heart (g)	.998 ± .045	1.16 ± .085	.792 ± .031*
Liver	14.1 ± .8	12.7 ± 1.0	10.8 ± .6
Brain	1.81 ± .04	1.82 ± .02	1.74 ± .03
Kidney	2.18 ± .1	2.27 ± .1	1.64 ± .06 *
Body wt	331 ± 16	343 ± 10	266 ± 7

* Significant at 99% confidence level compared to Group 2.

account for the overall decrease in size of gland. The cells of this zone were well outlined but cytoplasm and nucleus were reduced in size. The zona reticularis appeared relatively broad with deep penetrations of cells into the central connective tissue mass. The thyroid gland was composed of dilated acini surrounded by tall columnar cells. The colloid content of these acini was diminished with frequent resorption vacuoles at periphery. Papillary infolding was prominent. In the pituitary, the number of both eosinophil and chromophobe cells was reduced. Eosinophil cells were degranulated. Basophils appeared to make up about one-half of cells of the pituitary. These cells were swollen and stained only faintly with aniline blue. In more than half of these basophils (Fig. 5) the cytoplasm

showed a honeycomb structure containing numerous vacuoles. Some basophils were ballooned out so that outlines were no longer visible. In a small percentage large distinct hyalin globules were found.

Discussion. It is clear from these results that PTU treatment exerts a depressant effect on development of adrenal-regeneration hypertension, thus indirectly confirming results previously reported by Skelton(3) after thyroparathyroidectomy. It would be of interest to know whether the effect shown on blood pressure is accompanied by a favourable influence on development of cardiovascular and renal lesions. However, as we have reported earlier(5), the incidence of these lesions in our studies is less than that reported by Skelton(8). In this experiment no cardio-

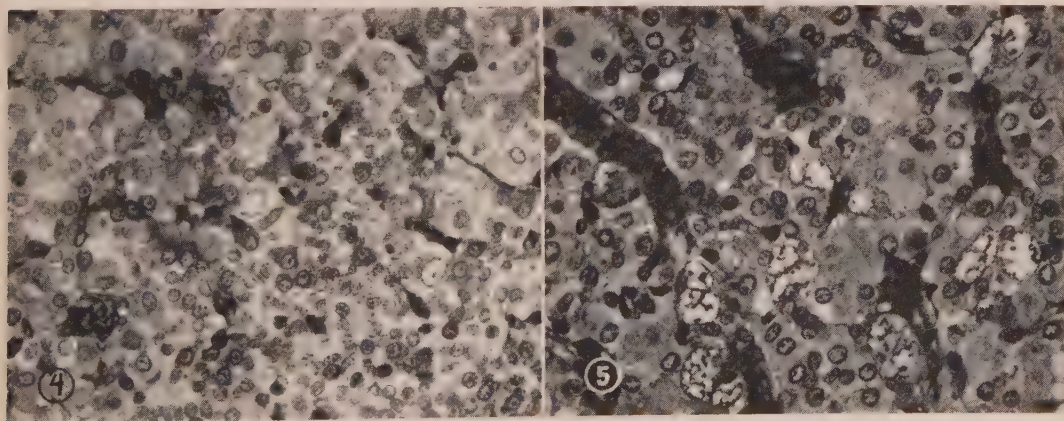


FIG. 4. Pituitary of adrenal hypertensive rat. Small cells with scanty pale cytoplasm are chromophobes. Darkly stained cells are eosinophils. Swollen large cells with occasional vacuoles are basophils. Rona-Morvay stain, $\times 310$.

FIG. 5. Pituitary changes in animal from Group 3 treated with PTU: marked hyperemia, large, vacuolar basophils and degranulation of eosinophils. One basophil at left, below a capillary, contains rough hyaline granules. Rona-Morvay stain, $\times 310$.

vascular or renal lesions were found in PTU treated animals, but since only 50% of adrenal-regeneration hypertensive controls developed such lesions no definite conclusions may be drawn from groups of animals of this size.

It is evident that with exception of weights of pituitary and thyroid glands, there is a general reduction in tissue weights in the PTU treated group. The generally lower weight of animals treated with antithyroid drugs is well known(9,10) and probably plays a large part in causing decrease in tissue weights. Although adrenal weights of PTU treated animals are not significantly lower than hypertensive controls when expressed in terms of body weight definite histological differences are seen when adrenals from these groups are compared. The shrinking of the fasciculata zone which we have observed has been reported by Deane and Greep following thiouracil therapy(1).

We reported earlier that Amphenone therapy, which lowers blood pressure of adrenal-regeneration hypertensive rats, affects primarily the fasciculata zone(5). Since Amphenone also possesses antithyroid properties (11), the possibility exists that the effects of this drug and PTU on blood pressure are mediated through a common mechanism. The histological picture of the pituitary gland, which we found after PTU treatment of rats with regenerated adrenals, is similar to that described by many workers after antithyroid drugs or thyroidectomy(12,13). However, the pituitary changes in control rats with adrenal-regeneration hypertension has not been reported. The basophilism in these animals may reflect a hyperactivity of the pituitary in response to hypofunction of the adrenal gland after unilateral adrenalectomy and contralateral adrenal enucleation. Available histochemical methods do not allow a differentiation between pituitary basophils

responsible for thyroid-stimulating and adrenal corticotrophic hormones and it is not known whether these functions reside in the same cells(13,14). It is hoped that studies in progress may allow us to throw some light on the role of pituitary factors in adrenal-regeneration hypertension.

Summary. Treatment of rats having adrenal-regeneration hypertension with propylthiouracil, results in decrease in body and tissue weights and marked lowering of hypertensive blood levels. This treatment also results in shrinking of width of the fasciculata zone in regenerated adrenal gland and a marked change in pituitary basophils. An increase in basophils of the pituitary has also been found in control rats with adrenal-regeneration hypertension.

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Ineffectiveness of Factor 3-Active Selenium Compounds in Muscular Dystrophy of Rabbits on Vitamin E-Free Diets. (23928)

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It has been reported(1) that the standard assay for tocopherol, using resorption sterility in the rat, is not influenced by Factor 3[†], even when the latter is supplied in large excess. This factor, or Factor 3-active selenium compounds, prevents muscular degeneration in rat,[‡] mouse(2) and also delays "white striation" of muscles in the chick (personal communication); all these lesions occur on *Torula* yeast diets deficient in Factor 3 and Vit. E. Recently, results have been described showing, on the other hand, that intramuscular injections of 20 and 100 μ g of selenite-selenium had no effect on nutritional muscular dystrophy of Vit. E deficient rabbits(3). We have found that muscular dystrophy in rabbits, when produced by Vit. E deficient soybean meal diet, is not preventable by selenocystine or sodium selenite, both of which are potent sources of Factor 3 activity. By rat assay against liver necrosis the soybean meal was shown to be a relatively good source of Factor 3. Determination of selenium in soybean meal by activation analysis produced values well in agreement with the bioassay.

Methods. Factor 3-activity of selenium compounds used was determined by rat assay against necrotic liver degeneration as described elsewhere(4,5). Selenium levels in soybean meal were measured by activation analysis (production of specific radioisotopes of selenium by neutron bombardment, and subsequent carrier analysis)(6). The basal diet for production of Vit. E deficiency and acute muscular dystrophy in rabbits has been described briefly(7). Its per-

centage composition is: soybean meal (solvent extracted, 51% protein), 40; cellulose (non-nutritive fiber of General Biochemicals), 10; sucrose, 32; salt mixture (Hubbel, Mendel, Wakeman) with 0.5% added ZnCO_3 , 5; B-vitamin premix, 5; lard (Vit. E-free of Distillation Products Industries), 6; and cod liver oil, U.S.P. 2. Vitamin premix contributed the following in μ g/g diet: thiamine, riboflavin and pyridoxine, 5 each; calcium pantothenate, 25; i-inositol, 200; niacin, 40; methyl 1,4-naphthoquinone, 0.3; folacin, 2; Vit. B₁₂, 0.03; and choline chloride, 2000. No Vit. E was added to the diet. Where desired, Vit. E was supplied to rabbits as 10 mg dl, α -tocopheryl acetate given orally once a week by calibrated dropper as 10% solution in olive oil U.S.P. Two sources of Factor 3-activity have been tested, *i.e.*, sodium selenite and D,L-selenocystine hydrochloride. The selenite was added to basal diet as a premix in sucrose such that addition of 1% of premix furnished 100 μ g% (1 ppm) of selenium. Selenocystine was added to the basal diet as a solution such that 5 ml/kg diet furnished 50 μ g% (0.5 ppm) of selenium. Male albino rabbits at 4 weeks of age were distributed among 4 treatment groups as follows: basal diet Vit. E-free; basal diet with Vit. E supplementation; Vit. E-free basal diet with Se as sodium selenite; and Vit. E-free basal diet with Se as selenocystine. Each group had 5 animals. Diets and tap water were given *ad libitum*. Animals were housed individually on raised, half-inch screens, and were weighed 3 times each week. Individual growth curves were plotted. The rabbits were checked daily for gross muscle weakness. Muscular dystrophy was noted on a 0-4 scale; grade 1 was the typical "duck waddle"; grade 2, inability to right itself when placed on its side; grade 3, inability to remain upright when placed upright; grade 4, terminal, immobilized condition, with complete and rather

* Nat. Inst. of Neurological Diseases and Blindness.

[†] Since various compounds of selenium show greatly different biological activity, the term "Factor 3" is used to designate the biologically active, selenium-containing component, or components, present in nutrients and other materials of biological origin.

[‡] Schwarz, K., to be published.

TABLE I. Lack of Effect of Factor 3-Active Selenium Compounds on Muscular Dystrophy in Rabbits Fed a Vit. E Deficient Diet (5 Rabbits/Group).

Supplement	Dose level of selenium ($\mu\text{g}/100\text{ g diet}$)	Factor 3 (Fischer units*/100 g diet)	Initial body wt (g)	Avg body wt increments (g) in 10-day periods			Days to clinical muscular dystrophy	Days to death
				0-10	10-20	20-30		
dl- α -tocopheryl acetate (10 mg/wk)			620	+310	+340	+260		
None			620	+300	+280	-190	24.6 (21-31)	37.1 (34-41)
Na selenite	100	730	640	+356	+160	-110	26.6 (21-31)	33.5 (23-39)
D,L-selenocystine HCl	50	345	610	+322	+184	-23	25.8 (20-36)	34.0 (23-42)

* 1 Fischer unit is amt required daily/animal by Fischer strain of rats to achieve 50% protection against liver necrosis. For details concerning these tests see reference (8).

spastic paralysis. When moribund or dead, dystrophic animals were examined for gross lesions of muscle, liver, heart and other organs. Portions of liver and leg muscles were saved for possible subsequent histologic evaluation.

Results. Rabbits fed the basal diet without Vit. E developed muscular dystrophy in about 3 weeks (Table I). The dystrophy progressed rapidly through various stages and terminated in death an average of 12 days after onset. All 5 animals in the group responded similarly. The 5 animals supplemented with Vit. E grew at a normal rate of over 30 g/day and remained in perfect health. Previous experience has shown that this diet, with Vit. E, maintained rabbits in a normal state for at least a year. Neither the selenite nor the selenocystine supplements to the basal Vit. E-free ration altered the course of dystrophy development in any rabbit fed these diets.

Under our experimental conditions muscular dystrophy in rabbits is caused by Vit. E deficiency alone and not by simultaneous lack of both Factor 3 and Vit. E. Tests for Factor 3 in soybean meal, using the rat assay gave the following results:

% soybean meal in diet	$\mu\text{g } \% \text{ Se supplied by soybean meal}$	% protection
5	1.5	35
10	3	46
20	6	100

Soybean meal, main component of rabbit diet, thus supplies Factor 3 at levels which

appear sufficient to obviate the need for additional supplies of Factor 3-active selenium compounds. The results of bioassays are borne out by activation analysis for selenium: 1 g of soybean meal contains 0.3 μg of the element, *i.e.*, at least 12 $\mu\text{g } \%$ of biologically active selenium are present in the basal, 40% soybean meal ration. In the rat necrotic liver degeneration will be prevented by 1.4-1.6 $\mu\text{g } \% \text{ Se}$ in form of α -Factor 3 concentrates(8).

It is obvious from these results that Factor 3-active selenium compounds do not substitute for Vit. E. There also does not appear to be a Vit. E sparing effect, as indicated by rate of development of muscular dystrophy and by time of death. It cannot be concluded, on the other hand, that Factor 3 deficiency would not impair structure and function of muscles, since the diet used for the production of muscular dystrophy contains sufficient amounts of the factor.

Summary. Muscular dystrophy developed within 3 weeks in young rabbits fed a basal Vit. E-free diet which contained soybean meal as the protein source. Sodium selenite, and selenocystine, when added to the basal diet as sources of Factor 3-activity, did not influence the course of the disease. Control rabbits fed the basal diet but supplemented with α -tocopheryl acetate grew at normal rate of over 30 g/day and remained in good health. Factor 3 assays in rats, and also activation analysis for selenium, established that an adequate level of Factor 3 was present in the basal soybean meal diet. Under our condi-

tions, muscular dystrophy is due to lack of tocopherol alone. Factor 3-active selenium compounds do not substitute for Vit. E.

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Urinary and Fecal Excretion of Orally Administered Mg^{28} * (23929)

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It is well recognized that magnesium is a biologically important ion. Studies of its metabolism, however, have until recently been hampered by technologic difficulties. Within the past several months a synthetic radioactive isotope of magnesium, Mg^{28} , with half-life of 21.8 hr, has become available and has made possible a reevaluation of metabolism of magnesium in human beings. The purpose of this pilot study was to follow the behavior of orally administered magnesium, using Mg^{28} as tracer. Urinary and fecal excretion of the isotope was measured.

Material and methods. Initial observations (Group A) were made in 12 hospitalized adult male convalescents from various diseases. None had any symptoms or signs of gastrointestinal disturbances, and all were on regular hospital diet. Radioactive magnesium (Mg^{28}) as magnesium chloride in an acid solution, was given in water, usually mid-morning. The dose of 10 to 25 μ c was contained in 3 to 10 meq of magnesium. All urine passed within next 24 hours was collected in chemically clean glassware. Seven patients were given

one dose of Mg^{28} ; 4 were given 2 doses a week apart; and the twelfth patient given 3 doses of tracer at weekly intervals. Subsequent studies (Groups B, C and D) were done in 14 healthy medical students. Each student received orally 10 μ c of Mg^{28} contained in 3 meq of magnesium. In 7 instances (Groups C and D) 2 gelatin capsules containing a gram of carmine were given at the same time. In addition to specimens of urine and feces collected in chemically clean glassware from all students, several venous blood specimens were obtained from 3 students in Group D. Two students (#11 and 12) were given a second dose of isotope one week after the first. Radioactivity of urine, plasma and feces was determined with a well-type scintillation counter and a scaling circuit. Stool specimens were digested in concentrated sulfuric and nitric acids prior to radioactivity assay. A total of 10,000 counts was made on each sample. All determinations were corrected for physical decay of isotope.

Results. The maximum 24 hour urinary excretion rate for Mg^{28} in hospitalized patients (Group A) was 6.27%; the mean, $2.11 \pm 1.61\%$. Except in one patient, the second and third determinations of urinary excretion rate gave results closely comparable to the first.

The 24 hour urinary excretion of radioactivity in the first 9 medical students (Group

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Mg^{28} was supplied by Brookhaven National Laboratory, Upton, L. I., on allocation from U. S. Atomic Energy Com.

[†] Established Investigator of Am. Heart Assn.

[‡] Fellow of U.S.P.H. Service.

TABLE I. Urinary and Fecal Excretion of Mg^{28} following Its Oral Administration.

Patient No.	% of dose recovered in urine				% of dose recovered in stool					
	0-24'	24-48'	48-72'	Total	0-24'	24-48'	48-72'	72-96'	96-120'	Total
Medical students										
Group C										
10	2.87	1.77			60.13	14.48	.65			75.26
11-1	4.27	2.72			.10	7.16	35.26	42.64		85.16
12-1	2.17	1.55			24.34	26.46		24.45		75.25
13	1.71	1.14			0	45.66	4.11	6.56		56.33
	2.76	2.39	Means							
Group D										
11-2	3.18	2.02	1.05	6.25	.22	1.56	11.67	32.26	37.08	82.79
12-2	3.98	3.28	.91	8.17	1.14	25.30			61.53	87.97
14	1.24	1.80	.17	3.21	41.62	14.28	3.50			59.40
	2.80	2.37	.71	Means						

B) was between 1.13 and 4.93% of total administered dose, mean value being $2.88 \pm 1.27\%$. Maximum total radioactivity recovered in stools within the first 48 hours was 61.85%.

Four other students (Group C) were then given, in addition to the same dose of Mg^{28} , the carmine indicator. Stools were collected for 96 hours, or until red color had disappeared. Urine specimens were collected for 48 hours. Urinary excretion of radioactivity during the first 24 hours was comparable to that observed in Groups A and B; excretion of Mg^{28} during the second 24-hour period was consistently lower than that during the first 24 hours. Maximum fecal recovery rate obtained in this group was 85.16% (Table I). Radioactivity was usually greatest in stools with most intense red color.

In the final set of observations (Group D), 3 students were given the same tracer dose of Mg^{28} and the carmine dye, but urine collections were extended over 72 hours and fecal collections through 120 hours. Urinary excretion of Mg^{28} during the first 48 hours was comparable to that observed in Group C; amount of Mg^{28} excreted during 48-72 hour period was lower than that observed in either of the preceding 24-hour periods. Maximum percentage of radioactivity recovered in stools was 87.97. In one subject (#12-2) 96% of radioactivity administered was recovered in stool and urine.

Blood specimens were obtained at 2, 4, 6 and 8 hours after oral administration of Mg^{28} to 3 students in Group D. Calculations, based

on specific activity of material administered, showed that rise in serum magnesium concentration attributable to orally administered material was on the order of 0.75 to 3.85×10^{-5} meq/l; the highest concentration in each subject was found at 4 hours (Table II).

Comments. In the therapy of eclamptogenic toxemia, Pritchard(1) reported that oral administration of approximately 300 to 600 meq of magnesium sulfate increased urinary excretion of magnesium by less than 5% of injected dose. Previous studies had shown that when magnesium sulfate is administered *parenterally* it is excreted solely by the kidneys, 70 to 90% being so eliminated(2). There was no evidence that any of the injected magnesium was excreted by way of the feces(3).

The results of our study are compatible with those reported by Pritchard(1), and extend his observations. Urinary excretion of a single dose of orally administered Mg^{28} was less than 10% and did not appear to be complete within 72 hours, although maximal excretion occurred during the first 24 hours. Since it has previously been shown that almost the entire dose of *parenterally* adminis-

TABLE II. Plasma Radioactivity Concentration following Oral Administration of Mg^{28} .

Subject	Hr after oral ingestion			
	2	4	6	8
11-2	1.05*	3.85	3.46	2.73
12-2	1.10	2.26	1.96	1.07
14	.75	1.12	1.09	

* All values = $\times 10^{-5}$ meq/l and are based on specific activity of material administered.

tered magnesium is excreted by the kidney, one may conclude that the low urinary excretion of orally administered Mg^{28} is due to poor gastrointestinal absorption of this material. The very slight rise in serum magnesium concentration following oral dose of magnesium also suggests poor gastrointestinal absorption, as does the recovery of 83 and 88% of total administered dose in the feces in the 2 medical students (#11 and 12).

Summary. A tracer dose of Mg^{28} (10 to 25 μ c contained in 3 to 10 meq of magnesium) was administered orally to 26 subjects, and serial specimens of blood, urine and feces were assayed for radioactivity. Urinary excretion within 72 hours accounted for less than 10% of total dose of Mg^{28} ; the maximal excretion (6.3%) occurred during the first

24-hour period. Maximal concentration of radioactivity in plasma occurred at 4 hours, but the actual increase in serum magnesium was negligible. Fecal excretion within 120 hours accounted for 60 to 88% of administered dose. Low renal excretion of magnesium is believed due to poor gastrointestinal absorption of this material.

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Effect of Experimentally Induced Fever on Alimentary Lipemia. (23930)

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The discovery of Hahn(1) that intravenous injection of heparin rapidly clears the turbidity of alimentary lipemia has been amply confirmed. Clearing occurs similarly when the serum of heparinized animals is mixed with lipemic serum and with fat emulsions *in vitro*, but not when heparin is added directly(2). This observation led to the conclusion that heparin does not act as such, but produces a clearing factor in the body. Release of heparin by shock and by histamine injection has a similar clearing effect(3).

The fortuitous observation that plasma of a patient with familial hyperlipemia cleared repeatedly during several bouts of fever caused by idiopathic pericarditis suggested that a rise in body temperature may similarly release the clearing factor. It was, therefore, attempted to investigate the possible effect of experimentally induced fever on alimentary lipemia in the dog.

Material and method. A) *In vivo studies.*

105 mongrel dogs of either sex, weighing 10 to 15 kg were fasted for 48 hours. The animals were then fed 10 egg yolks either alone or added to a mixture of small pieces of cooked meat and oat meal. To prevent regurgitation each animal received 50-60 mg of Thorazine intramuscularly immediately after eating. The animals were divided into 6 groups: 1) 25 dogs serving as controls were fed the egg yolk meal as indicated above. 2) 30 dogs received 0.5-0.7 cc of typhoid and paratyphoid vaccine (Parke, Davis and Co.) subcutaneously. 3) 11 animals were given 100 γ of Piromen (Travenol Laboratory, Inc.)[†] intravenously 1½ hours after test meal. 4) 25 dogs were injected with 25 mg of heparin intravenously after similar time interval. 5) 8 dogs received simultaneously injections of 0.5-0.7 cc of typhoid and paratyphoid vaccine subcutaneously and 25 mg

[†] Piromen is non-protein, bacterial component, containing mixture of nucleic acids and polysaccharide complex.

* Deceased.

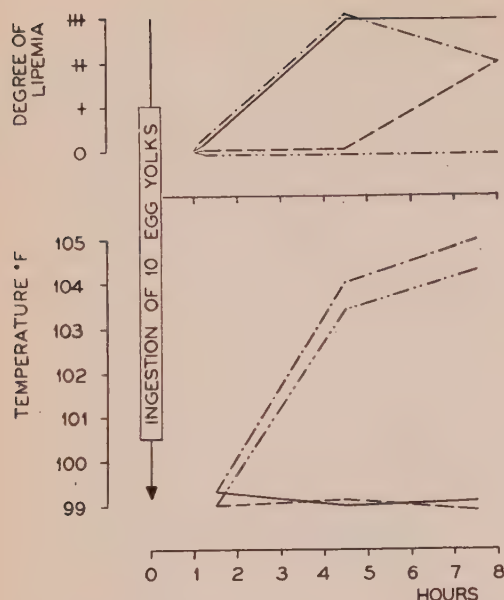


FIG. 1. Effect of vaccine alone or simultaneously with protamine sulfate, and heparin on body temperature and alimentary lipemia of 3 dogs after ingestion of 10 egg yolks. Control animals received no medication after the test meal.

— · · · · 0.7 cc typhoid and paratyphoid vaccine;
 — — — 0.7 cc typhoid and paratyphoid vaccine +
 25 mg protamine sulfate; — — — 25 mg heparin;
 ————— Control.

protamine sulfate intravenously 1½ hours after test meal. 6) 6 dogs received a single subcutaneous injection of 0.7 cc of typhoid and paratyphoid vaccine daily for 8 days. All animals of this group received a test meal on first and last day of experiment 1½ hours prior to injection of the vaccine. Rectal temperature of all dogs was recorded at one-half hour intervals for 6-8 hours after ingestion of egg yolk meal. Blood samples were withdrawn and immediately centrifuged at similar periods. After separation from the clot the turbidity of the sera was estimated and graded from 0-3 +. B) *In vitro* studies. To investigate *in vitro* the effect of temperatures equivalent to those *in vivo* on lipemia, sera of 8 dogs obtained from 1½ to 4 hours after the egg yolk meal were placed in water bath of 105° up to 24 hours.

Results. A) *In vivo* experiments. 25 dogs receiving vaccine and 8 receiving Piromen developed a marked rise of temperature within 1½ hours varying from 103 to 107°F which

remained significantly elevated to 7 hours after injection. None of the animals of the other 5 groups had increase of temperature.

All dogs developed marked lipemia 1½ hours after ingestion of test meal. Sera of control animals remained turbid throughout. However, lactescence had disappeared when blood was drawn 24 hours later. Sera of those dogs in whom administration of the vaccine or of Piromen produced fever of 103°F or more cleared rapidly and remained so for the following 8 hours (Fig. 1), while no change of lipemia occurred in those animals with temperature below that level. Administration of heparin induced immediate clearing of the alimentary lipemia. After 3 hours, however, the sera became turbid again and remained so until end of experiment (Fig. 1). Administration of protamine sulfate to vaccine-treated dogs prevented clearing of heavy lipemia despite a marked temperature rise. Daily administration of typhoid and paratyphoid vaccine for one week failed to induce significant temperature elevations from 3rd day on. Complete clearing of the lipemic sera occurred after test meal on first day when the temperatures rose above 104°F, while it was not observed on repeating the experiment on 7th day.

B) *In vitro* experiments. Turbidity of the sera obtained from dogs fed egg yolk meal, failed to clear when kept in water bath at 105°F up to 12 consecutive hours.

Discussion. Our experiments demonstrate that administration of typhoid and paratyphoid vaccine as well as Piromen caused rapid clearing of alimentary lipemia in dogs. This phenomenon appears to be unspecific and apparently associated with marked rise in temperature since no clearing of the turbidity occurred in animals which remained afebrile or whose temperature elevation never exceeded 103°F. Prevention of clearing of the lactescent serum by injection of protamine sulfate simultaneously with vaccine suggests that the fever, which rose to above 103°F, may cause release of heparin or a heparin-like substance to produce the clearing factor. This hypothesis is based on observations that injection of protamine sulfate, known to combine with and inactivate heparin(4), reverses the clear-

ing of alimentary lipemia in dogs and rats (5). Furthermore, administration of heparin caused also lipemia clearing although of shorter duration. The assumption that not the high body temperature *per se* but the production of the clearing factor by an intermediary mechanism, possibly release of heparin, induced by the fever is necessary for clearing of the lipemic sera is also suggested by the *in vitro* experiments in which lactescent sera of dogs fed the egg yolk meal failed to clear when kept in water bath of 105°F for 24 hours, a temperature approximately equivalent to that obtained in animals after vaccine and Piromen injection.

Summary. Administration of typhoid and paratyphoid vaccine as well as Piromen caused rapid clearing of alimentary lipemia in dogs which developed fever of 103°F or more. This phenomenon appears to be un-

specific and seemingly due to marked temperature rise induced by these agents, since no change of turbidity occurred in animals with fever below 103°F. Administration of protamine sulfate to vaccine-treated dogs prevented clearing of the lipemic sera despite a marked elevation of temperature. No clearing of turbidity occurred when the lactescent sera were kept in water bath at 105°F for 24 hours. It is suggested that high body temperature in the dog may cause release of heparin or a heparin-like substance to produce a lipemia clearing factor.

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Increased Serum Glutamic-Oxalacetic Transaminase of Monkeys Following Oral Administration of Staphylococcal Enterotoxin.* (23931)

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The symptoms of staphylococcal food poisoning are nausea, vomiting, retching, abdominal cramping of varying severity, and diarrhea after an incubation period of 1-6 hrs. Marked prostration with hypothermia and hypotension may occur(1). However, no histopathology has been observed in animals fed enterotoxin.

This paper reports on serum glutamic-oxalacetic transaminase (GOT) levels of monkeys following oral administration of staphylococcal enterotoxin. Elevation of serum GOT which occurs in several disease states(2,3) is attributed to release of the enzyme into the blood circulation following death of injured cells or increase in cellular membrane permeability(4). The slight, but distinct, rise in serum GOT of monkeys fed enterotoxin suggests cellular injury.

* This work was supported by contributions from various companies and associations of food industries.

Materials and methods. Blood samples were obtained from a leg vein just before enterotoxin was given by stomach tube. These prefeeding blood samples were used to establish normal GOT levels of individual animals. Animals were observed for 5 hrs, vomiting being the criterion of the reaction of monkeys to enterotoxin. Sensitivity of monkeys to enterotoxin varied considerably, some being used for the first time, others having developed substantial resistance through previous feedings of enterotoxin(5). Post-feeding GOT levels were determined on blood drawn about 6 hrs after toxin feeding. GOT assays were made on unhemolysed sera by a method essentially identical to Steinberg *et al.*(6). The Beckmann DU spectrophotometer was used; units are corrected to incubation at 25°C. Sera prepared the day of toxin feeding were kept frozen until used. Sera whose GOT values were to be compared were assayed the

TABLE I. Serum GOT Units Immediately before and 6 Hours after Feeding Enterotoxin and Water.

Monkey	Times vomit	Enterotoxin		Water	
		Before	After	Before	After
1	0	28	61(218)*	28	28
2	3	23	54(235)	22	25
3	1	34	78(239)	39	42
4	1	29	68(232)	33	33
5	0	19	37(195)	18	19

* % of titer before feeding.

same day. Duplicate tests were made with some sera; variations of greater than 4 units were not encountered. Enterotoxin samples which may be antigenically different were prepared from 2 different *Staphylococcus* strains (5). The preparations varied in purity(7), but dosages used were judged capable of causing emesis in about one-half of the animals.

Results. Typical results obtained with "new" monkeys (animals being fed enterotoxin for first time) are presented in Table I. 50 μ g of an enterotoxin preparation of 20-25% purity were fed to each animal. Normal variation of GOT was determined after feeding of water on the same group of monkeys 2 weeks after enterotoxin feeding.

Six hrs after enterotoxin is fed to monkeys serum GOT increased approximately 2-fold. Diurnal variation and the effect of handling of animals is insignificant. Similar control experiments of feeding water showed elevation of GOT up to 130% of the original level; one animal out of 24 had a titer 150% above the prefeeding level. Normal GOT titers of monkeys are generally within normal range of GOT values for humans. In our experiments relative increase seems more significant than absolute values since GOT titer immediately before toxin administration is used as the reference value. On this basis, a rise of GOT above 175% of the prefeeding titer has been considered significant.

GOT reached its maximum at about 6-8 hrs after enterotoxin administration and dropped considerably by 24 hrs. Table II shows GOT values of serial samples taken over a 24 hr period. This experiment was done after it had been established that GOT was slightly higher at 6 than at 4 hrs.

The magnitude of GOT rise did not show a

correlation with the number of times the animals vomited. Approximately 40% of 28 monkeys which were fed enterotoxin but which did not respond with emesis showed significant GOT elevation. On the other hand, about 15% of 29 monkeys in which vomiting was provoked by enterotoxin showed no significant rise of titer. The number of "new" monkeys available was limited in number. However, regardless of whether enterotoxin induced vomiting or not, these animals reacted much more consistently with significant GOT elevation than animals which had become partially resistant to the toxin. In fact, the greater majority of these "new" animals developed titers from 190-300% of the prefeeding GOT values.

Further evidence that GOT increase is a specific reaction to enterotoxin is illustrated in Table III. Even 3 mg/monkey of a control preparation derived from a non-enterotoxigenic *Staphylococcus* strain by essentially the same purification procedure as the toxin sample of experiment of Table I produced no change in GOT. In contrast, 20 μ g/monkey of essentially pure enterotoxin (Strain S-6) induced significant elevation as did 1 mg/monkey of another antigenically different enterotoxin preparation (Strain 196-E).

The nature of response of animals to enterotoxin would indicate fluid loss. This decrease in blood volume does not explain the GOT rise. Prefeeding and 6 hr postfeeding hematocrit values(8) of blood of 3 monkeys fed enterotoxin (emesis in 2) were 44 and 48%, 45 and 50% and 43 and 40%, respectively.

Enterotoxin preparations possess no GOT activity of their own. Addition of enterotoxin to human or monkey serum did not increase GOT activity. Appreciable coagulase

TABLE II. Serum GOT Units Immediately before and at Different Time Intervals after the Feeding of Enterotoxin.

Monkey	Times vomit	Before feeding	Hr after feeding		
			6	10	24
1	(control)	34	38	39	42
2	0	34	76	66	42
3	0	30	36	38	27
4	3	31	90	57	41
5	1	25	36	34	27
6	0	27	67	65	40

TABLE III. Serum GOT Units Immediately before and 6 Hours after Feeding of Non-enterotoxic Control and 2 Antigenically Different Enterotoxin Samples.

3 mg/monkey, control			20 μ g/monkey, S-6			1 mg/monkey, 196E		
Times vomit	Before	After	Times vomit	Before	After	Times vomit	Before	After
0	21	23	1	30	54(190)*	1	32	61(191)*
0	31	31	5	39	91(260)	4	34	70(206)
0	23	22	0	38	76(200)	1	30	59(197)
0	23	26	0	21	53(252)	0	24	36(150)
0	30	29						

* % of titer before feeding.

and fibrinolysin present in some of the enterotoxin preparations necessitated GOT assay by a method whereby clarification of the reaction mixture was possible(9).

No significant changes in serum malic dehydrogenase, lactic dehydrogenase, and glutamic-pyruvic acid transaminase were found in monkeys fed enterotoxin.

Discussion. Elevated serum GOT reflects the enzyme release into the blood stream and is a measure of degree of tissue injury(4). In myocardial infarction and acute hepatocellular injury GOT may be 2-20 times or more above normal. The 2-3 fold increase of monkey serum GOT following oral administration of enterotoxin is small compared to that found in the above mentioned diseases, but this may be misleading. If the cells affected by enterotoxin are poor in GOT, then serum GOT rise may be limited. The suggested cellular injury by enterotoxin may be a secondary effect following primary action on the central nervous system; *e.g.* abnormal hypermotility of the gastrointestinal tract resulting in tissue damage. Presence of blood in the vomitus and stools of severe staphylococcus food poisoning cases is suggestive in this respect.

Some toxic reaction to enterotoxin other than emesis is needed before GOT increase occurs. All animals responding with vomiting may not necessarily react with sufficient severity in the required manner; this could explain absence of GOT rise even when animals vomit. Conversely, animals not vomiting after enterotoxin administration may still be reacting to the toxin; *e.g.*, 12.1% of 122 human staphylococcal food poisoning cases did not suffer from vomiting(10). Elevation of GOT could occur in these instances.

The transitory nature of GOT rise can be

explained by the short duration of staphylococcal food poisoning symptoms. Although the disease can be extremely uncomfortable the toxemia is seldom fatal, recovery is usually rapid, and no permanent injury results. Release of cellular GOT into the blood stream may occur only during the short acute stage of intoxication giving small, but definite, GOT increase with early maximum titer. The GOT picture is similar following X-irradiation(11).

The evidence presented indicates that increase of GOT is a specific response of the animal to enterotoxin. Since some of the animals not reacting to enterotoxin with emesis show elevated GOT, the monkey feeding test can be made more sensitive by GOT determinations on these animals.

A study should be made of the possible rise of GOT in staphylococcal food poisoning in man. So far, such an opportunity has not presented itself.

Summary. Serum glutamic-oxalacetic acid transaminase activity of monkeys after staphylococcal enterotoxin feeding has been studied. There is an elevation to about 2 to 3 times the prefeeding titer within 6-8 hrs after feeding of the toxin. The observations are discussed on the basis that increased serum transaminase activity is due to release of the enzyme into the circulation by damaged cells.

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Uterine Growth Promoting Action of Relaxin. (23932)

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Previous reports indicate that relaxin may have an effect upon several metabolic alterations of the uterus of rat, guinea pig and human. These alterations include glycogen and water content(1,2,3), decidual tissue reactions(4,5) and myometrial activity(6-11). We recently reported that large doses of Relaxin augmented wet and dry uterine weights of non-ovariectomized immature rats, and that exogenous relaxin potentiated weight response of uterus of ovariectomized immature rat to a physiologic level of estradiol-17β (11).

These observations raise the question as to the effect of different dosages of relaxin on relative contents of nitrogen, water and non-nitrogenous solids as well as gross wet and dry weights of uteri of immature female rats. The present study is directly concerned with ascertaining these effects.

Methods. Two groups of intact immature albino female rats were injected daily for 3 days and necropsied on the fourth, one group of females 33 days of age at start of experiment, the other group 28 days old. The animals were neither ovariectomized nor otherwise treated so as to change the hormonal balance.

In each of the 2 age groups there were 8 subgroups receiving saline or different doses of relaxin† as shown in the table. The 3 daily doses were given subcutaneously in 0.50 ml fluid. At necropsy the animals were weighed, uteri dissected, blotted on paper, weighed, dried at 110°C for 24 hours, and reweighed. The nitrogen content of the dried uteri was then determined by the Kjeldahl method.

Results. Wet and dry uterine weights are summarized in Table I. Weights of animals

TABLE I. Weight Responses to Relaxin of Uteri of Rats not in Estrus Necropsied at 36 Days of Age and 31 Days of Age.

Treatment, total amt Relaxin in mg	Age in days							
	31	36	31	36	31	36	31	36
	No. of rats		mg % wet wt		mg % dry wt		% water content	
Saline controls	10	15	45 ± 3.5	47 ± 2.6	7.4 ± .6	7.9 ± .5	85	85
.0002	7		47 ± 1.5		8.3 ± .6		82	
.001	10	10	51 ± 2.4	47 ± 4.6	8.1 ± .8	7.8 ± .6	86	83
.01	10	9	52 ± 2.4	47 ± 3.5	8.3 ± .6	7.1 ± .7	86	85
.1	17	8	58 ± 3.3	50 ± 4.6	9.7 ± .6	8.5 ± .7	85	85
1.0	10	7	64 ± 4.1	52 ± 4.6	8.4 ± .6	9.8 ± 1.2	87	86
2.5		15		58 ± 3.2		10.7 ± .6		82
5.0	10	7	63 ± 6.0	66 ± 4.2	10.1 ± 1.1	11.4 ± .9	84	86
10.0	8	9	67 ± 7.0	70 ± 3.9	10.6 ± 1.6	11.5 ± .9	84	83

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‡ The authors wish to acknowledge generous supplies of relaxin (Cervilaxin®) furnished by The Princeton Laboratories, Princeton, N. J.

TABLE II. Influence of Relaxin on Nitrogen Content of Uteri of Rats Necropsied at 31 and 36 Days of Age.

Treatment, total amt Relaxin in mg	31 days 36 days		31 days 36 days	
	No. of rats		Nitrogen content of dry wt, %	
Saline	10	12	14	13
.001	10	10	15	13
.01	10	10	12	14
.1	10	10	12	12
1.0	10	10	13	13
2.0	10		14	
5.0	10	10	16	13
10.0	8	10	16	12

which had prematurely entered estrous cycles are not included in the averages in this table. Uteri of younger rats showed a greater relative increase in growth than older ones, especially in low dosage ranges. Older animals developed heavier uteri as shown by dry weights after minimum dosage of 0.1 mg, whereas uteri of younger animals responded measurably to 0.0002 mg. Thus it seems that rats 28 to 31 days of age are sensitive to extremely small doses of relaxin. As body weights of animals killed at 36 days of age average 82 g while those killed at 31 days averaged 66 g, the absolute uterine weights of the older animals were greater than those of younger rats for each given dosage.

Nitrogen determinations were done mainly on groups of 10 uteri from each of the original groups regardless of whether or not they manifested vaginal estrus, except for the 10 mg relaxin-treated 31-day-old group in which the results of 2 animals abnormally in estrus are not included.

Results of water and nitrogen determinations thus show a rather constant percentage of each within the limits of experimental error, regardless of age of animals or treatment received. Younger animals exhibit this constancy rather well, while older ones show it less significantly. It may thus be concluded that the increase in uterine weight seen in treated animals after 3 days of relaxin injection is not due to preferential increase in one

of these factors, but rather represents a uniform increase in all components.

Summary. Experiments were performed to determine response of uteri of immature albino rats to subcutaneous injections of varying dosages of relaxin. Saline and 0.0002, 0.001, 0.01, 0.1, 1, 5, and 10 mg relaxin were injected into female rats 28 and 33 days old by dividing the dose into thirds and administering each third on 3 consecutive days. Necropsies were performed on the fourth day at ages 31 and 36 days, respectively, and the uteri weighed, dried and reweighed. Wet uterine weights, *i.e.* mg % of body weight, increased along a sigmoid pattern from 45 for saline to 67 for 10 mg in younger group, and from 47 for saline to 70 for 10 mg in older group. Water concentration remained relatively constant, varying between 82 and 87% of wet weights, with no correlation as to amount of relaxin received. Nitrogen content showed a similar response, ranging between 12 and 14% for 36-day-old animals, and 12 and 16% for 31-day-old animals.

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Measurement of Osmotic Fragility of Fibroblasts. (23933)

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Studies of physical properties of a number of different types of cell membranes have led to a proposed basic structure for the cell membrane, described as consisting of continuous bimolecular film of lipid with a layer of protein adsorbed on both surfaces(1,2,3,7). The "elasticity and great mechanical strength" of the membrane have been attributed to cross-linked polypeptide chains in these protein layers(4). In such a membrane it might be expected that hydrolysis of polypeptide links in the outer protein layer would reduce the mechanical strength of the membrane. Experiments described below attempted to demonstrate this by subjecting samples of fibroblasts to 2 proteolytic enzymes, trypsin and papain, and measuring osmotic fragility of treated and untreated cells. It was assumed that a decrease in mechanical strength of the membrane would be reflected by increase in osmotic fragility of the cells. Trypsin was used because of its well known dispersing effect on fibroblasts and other cells grown *in vitro*, suggesting activity at cell surface. Papain was used because of its reported ability to digest certain living cells(5,6). A comparison is also made between osmotic swelling in fibroblasts and in erythrocytes. The latter swell in a manner suggesting that erythrocyte cell membrane is a nearly inexpandible structure, which permits an increase in cell volume by changing in shape from disc to sphere and not by increasing in surface area(8,9). In contrast to this, observations show (described below) that the fibroblast cell membrane is capable of considerable expansion in area. This, along with the relative rigidity of form of the erythrocyte membrane, even after hemolysis(10) suggests basic structural differences between erythrocyte and fibroblast cell membranes.

Methods. Osmotic fragility measurements on fibroblasts. Cells were obtained from 5-

day-old cultures of Earle's strain L mouse fibroblasts grown *in vitro* in medium consisting of 40% horse serum, 40% Hanks solution, and 20% chick embryo extract, plus penicillin and streptomycin. The cells were centrifuged from culture medium for 5 minutes at 1,000 rpm and then rinsed twice in Hanks solution (containing no phenol red or other indicator). The packed cells were then diluted 1-10 by volume in isotonic Hanks solution, and 0.10 ml aliquots of resulting suspension were added to tubes containing 3½ ml each of Hanks solution in dilutions ranging from isotonic strength (100%) to distilled water. After thirty minutes exposure the tubes were centrifuged and supernatant solutions ("A") were saved. The cells in each tube were then resuspended in distilled water for 2 hours, centrifuged out, and supernatants ("B") again saved. The degree of lysis was determined by measuring optical density at 260 mμ of each pair of supernatant solutions, and computing the percent lysis at each dilution as

$$\frac{[\text{O.D.}] \text{ of "A"} \times 100}{[\text{O.D.}] \text{ of "A"} + [\text{O.D.}] \text{ of "B"}}$$

Results indicate a small amount of cell lysis at concentrations between 100% and approximately 13% isotonicity. This is probably due to mechanical effects of centrifugation and pipetting plus surface effects of glassware, which was not siliconized. Beginning

TABLE I. Absorption of Supernatant Solutions.

Final % isotonicity (% Hanks)	[Optical density] _{λ=260}		% lysis
	Supernatant A	B	
100	.041	.216	16
57	.046	.258	15
36	.020	.276	7
30	.023	.458	5
23	.028	.473	6
19	.044	.516	8
13	.070	.443	14
11	.127	.375	25
8	.256	.229	53
6	.388	.117	77
3	.422	.063	87

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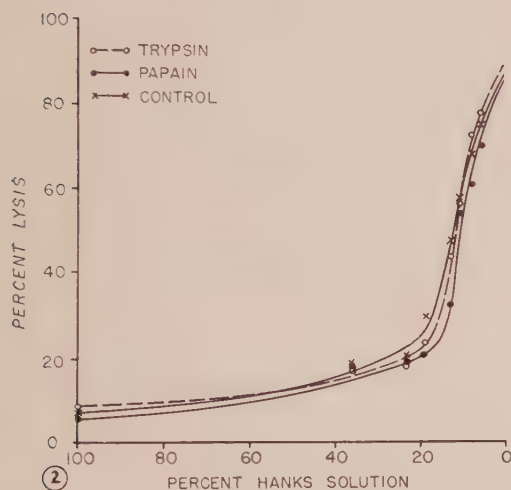
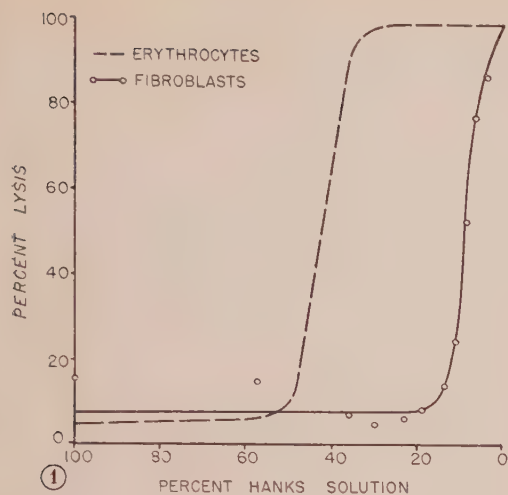


FIG. 1. Comparative osmotic fragility of erythrocytes (from Wintrobe¹²) and strain L fibroblasts.

FIG. 2. Effect of trypsin and papain on osmotic fragility of strain L fibroblasts.

at 13% the degree of lysis rises sharply as tonicity approaches zero. Similar results were obtained when tests were run at 5° instead of 37°C, and when cells were suspended in dilutions of sodium chloride solution instead of Hanks solution.

Absorption of supernatant solutions (which was maximal at 260 m μ) was presumed to be due primarily to RNA-protein released from cell cytoplasm(11). This is inferred from microscopic observation that the cell nuclei appeared to remain intact after osmotic disruption of the cytoplasmic membrane. Presence of relatively large amounts of cyto-

plasmic RNA has been demonstrated in one strain of fibroblasts grown *in vitro*, where the ratio of total cellular RNA to DNA was approximately 5 to 1 in 5-day-old cultures(13, 14). Direct chemical analysis of the supernatant solutions was not done.

Figure 1 shows percent lysis at each dilution of Hanks solution for strain L fibroblasts. For comparison a typical curve of erythrocyte osmotic fragility(12) is included, showing hemolysis beginning at approximately 50% tonicity.

Microscope observations of fibroblast swelling. Wet slide-coverslip preparations of strain L fibroblasts were made and examined at 1,000 X (phase contrast). When distilled water was put under edge of coverslip, each cell swelled rapidly to 3 or 4 times its original diameter. After a variable time, up to about 3 minutes, the cell membrane ruptured abruptly at a point, and the intracellular granules were extruded through the defect as the membrane contracted to its original size. Nuclei showed very little swelling and never burst. Microscopically it appeared that the cell membrane was expansile and that there was tension in the expanded membrane causing it to contract again after its rupture.

Effects of proteolytic enzymes on fibroblast fragility. Washed samples of strain L fibroblasts were suspended in solutions of either trypsin[†] or papain[‡] for 30 minutes at 37°C. The concentration used for both enzymes was 0.5 mg/ml. The cells were then washed twice in Hanks solution and subjected to the fragility test described above. Controls were treated in the same way except that in place of enzymes, plain Hanks solution was substituted. The results are shown in Fig. 2. In enzyme-treated samples and in controls, the curve rises sharply at a tonicity of approximately 13%. The elevated baselines probably represent increased mechanical fragmentation due to extra rinsing, pipetting and centrifugation involved in pretreatment with the enzymes.

[†] Thrice crystallized trypsin obtained from Worthington Corp., N. J.

[‡] Crystalline protease prepared from crude papain by Dr. Emil Smith (Dept. of Biochemistry, University of Utah).

The fact that enzyme treatment produced no increase in cell fragility may be interpreted in one of two ways: 1. There is no substrate for trypsin and papain in the concentrations used at the cell surface of strain L fibroblasts. 2. There is a protein substrate for these enzymes at the cell surface, which was hydrolyzed by the enzymes, but this protein does not play a significant role in imparting strength to the membrane (as measured by osmotic fragility).

Summary. 1. A method is described for measuring osmotic fragility of cells containing no visible pigment and available in limited quantities only. 2. By this method it was found that osmotic fragility of Earle's strain L fibroblasts is considerably less than that of mammalian erythrocytes, fibroblasts lysing at approximately 13% of isotonicity and erythrocytes at approximately 50%. 3. Microscopic observations are described suggesting that the cell membrane of Earle's strain L fibroblasts is an expansile structure. 4. Treatment of strain L fibroblasts with trypsin or papain produced no increase in their osmotic fragility.

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Effect of Vit. E-Deficiency and Dietary Glycine on Tissue Phosphatase Activity.* (23934)

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Carey *et al.*(1) reported that alkaline phosphatase activity of muscle homogenates from dystrophic rabbits was unchanged from that of control animals, whereas a 2-fold increase in muscle acid phosphatase activity from dystrophic rabbits was observed. White and Hess(2) found no alteration in serum phosphatase activity in children and adults afflicted with progressive muscular dystrophy. Tallan(3) showed an increase in level of most free amino acids in muscle extracts of dystrophic rabbits except for the concentration of glycine which was markedly reduced. This

was later confirmed by Smith and Nelson(4) who in addition showed that glycine was consistently reduced in 4 other tissues of dystrophic rabbits. It seemed desirable to determine what effect, if any, the dystrophic condition as well as dietary glycine would have on enzymic activity of tissue other than muscle. Our studies describe the acid and alkaline phosphatase activity in liver, kidney, heart, spleen, brain, and muscle of normal and Vit. E-deficient rabbits, and the influence of dietary glycine on phosphatase activity of these tissues.

Methods. Twelve male rabbits, divided into 4 equal groups, were fed the following diets *ad lib.*: A. Control diet 11 of Goettsch

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TABLE I. Acid and Alkaline Phosphatase Activity of Tissues from Normal and Vit. E-Deficient Rabbits. Values expressed as μM P released/g dry tissue and probable error of the mean.

Tissue	Alkaline phosphatase (pH 9.0)				Acid phosphatase (pH 4.5)			
	Diet A	Diet B	Diet C	Diet D	Diet A	Diet B	Diet C	Diet D
	Control	Deficient	Control + glycine	Deficient	Control	Deficient	Control + glycine	Deficient + glycine
Liver	102 $\pm$ 1.6	79 $\pm$ 4.2	104 $\pm$ 7.2	98 $\pm$ 19.5	122 $\pm$ 2.7	90 $\pm$ 2.6	123 $\pm$ 6.8	109 $\pm$ 4.1
Kidney	740 $\pm$ 77.8	371 $\pm$ 39.5	534 $\pm$ 3.1	691 $\pm$ 69.0	323 $\pm$ 46.6	209 $\pm$ 13.2	207 $\pm$ 7.2	234 $\pm$ 6.6
Spleen	675 $\pm$ 41.1	464 $\pm$ 78.2	579 $\pm$ 98.4	682 $\pm$ 47.0	638 $\pm$ 15.5	379 $\pm$ 29.0	528 $\pm$ 84.9	624 $\pm$ 10.4
Brain	123 $\pm$ 4.0	85 $\pm$ 1.2	100 $\pm$ 3.5	111 $\pm$ 1.4	111 $\pm$ 5.1	84 $\pm$ 3.2	62 $\pm$ 7.2	96 $\pm$ 14.8
Heart	146 $\pm$ 35.5	53 $\pm$ 3.0	68 $\pm$ 4.0	65*	233 $\pm$ 46.0	86 $\pm$ 3.8	106 $\pm$ 8.4	100 $\pm$ 2.0
Muscle	25 $\pm$ 5.8	23 $\pm$ 6.3	23 $\pm$ 3.8	25 $\pm$ 5.5	123 $\pm$ 8.8	84 $\pm$ 7.6	75 $\pm$ 1.8	83 $\pm$ 0.9

* Single determination.

and Pappenheimer(5); B. Dystrophy producing diet 11 which had been treated with 1% ethereal FeCl_3 (5); C. Control diet 11 plus 1% glycine; D. Dystrophy-producing diet 11 plus 1% glycine. Controls also received oral supplements of 4 mg of alpha-tocopherol acetate/kilo body weight twice weekly. When symptoms(4) appeared, the animals were sacrificed. All deficient animals developed dystrophy at about the same time although those receiving glycine supplement exhibited fewer outward signs. One of the deficient animals died after 6 weeks and was excluded from the study. As soon as animals were sacrificed, samples of tissue were immediately homogenized with Teflon pestle in distilled water. The incubation system for phosphatase activity contained in final volume of 11 ml, 9 ml of buffer (veronal-acetate, pH 4.5, or veronal, pH 9.0), 1 ml of substrate (15×10^{-3} moles of MgCl_2), and 1 ml of homogenate. Incubations were carried out in small Erlenmeyer flasks with shaking in constant temperature bath at 37°C . After one hour, the reaction was terminated by addition of 5 ml of 20% trichloroacetic acid. Inorganic phosphorus was determined in a similar manner except that substrate was omitted. A 1 ml aliquot of each homogenate was removed and kept at 100°C for 24 hrs for determination of dry weight.

Results. Distribution of acid and alkaline phosphatase activity in various tissues of normal and dystrophic rabbits is shown in Table I. Values are expressed as μM of P released/g of dry tissue, and represent average of tissues from 3 rabbits, except for 2 animals on Diet D. There was a definite decrease in both acid and alkaline phosphatase of tissues

of dystrophic animals on Diet B as compared to controls on Diet A. Kidney and heart tissue showed the largest decrease (50 and 64% respectively) at pH 9.0; whereas heart and spleen were most affected at pH 4.5 (63 and 40%). Only muscle homogenate at pH 9.0 showed no significant decrease. In contrast to these findings, tissue homogenates of animals on dystrophy-producing Diet D + 1% glycine showed no decrease in either acid or alkaline phosphatase activity as compared to their controls on Diet C.

Discussion. Most enzyme assays on Vit. E-deficient animals have naturally been carried out on muscle homogenates since this is the most obviously affected tissue. Jacobi *et al.* (6) found no differences in the activities of 6 enzyme systems between dystrophic and normal muscle. The same was true for uricase and adenosinetriphosphatase activities of liver homogenates from normal and dystrophic animals. Carpenter *et al.*(7) reported that dystrophic rabbit muscle has normal phosphoglucutase activity and glycolysis.

Our results are in agreement with Carey *et al.*(1) with respect to alkaline phosphatase; however we found a slight decrease in acid phosphatase. Furthermore our results showed a significant decrease in both acid and alkaline phosphatase activity of 5 other tissues of dystrophic animals. Perhaps our most striking finding was that dietary glycine prevented decrease in tissue phosphatase of animals on Vit. E-deficient diet. Ray & Sen(8) reported that starvation decreased phosphatase activity of tissues, and that several dietary factors, including glycine, were effective in its regeneration. It should be emphasized, however, that our animals were not starved,

even the dystrophic animals continued to eat and show a weight gain until their sacrifice.

It is recognized that there is a disturbance of creatine metabolism in Vit. E-deficient animals. As early as 1932(9) attempts were made, without success, to treat progressive muscular dystrophy by supplementation with glycine on the assumption that there was a disturbance in formation of creatine. Dinning and Fitch(10) have recently reported that rate of creatine synthesis and turnover rate of skeletal muscle creatine is increased in dystrophic animals. Nevertheless that glycine decreases in most tissues of dystrophic animals and dietary glycine appears to prevent a decrease in tissue phosphatase, even though this enzyme has not been implicated with the dystrophic state, suggests that glycine is in some unknown way implicated in the dystrophic state.

Summary. 1. Acid and alkaline phosphatase activity of 6 tissues of normal and Vit. E-deficient rabbits was determined. 2. These activities were decreased in heart, spleen,

liver, kidney and brain of dystrophic animals. 3. Acid phosphatase decreased slightly in muscle, but no change occurred in alkaline phosphatase activity. 4. 1% glycine in the dystrophic diet prevented reduction of tissue phosphatase.

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Enzyme and Nucleic Acid Content of Thrombocytes from Normal and Thrombocytopenic Calves.* (23935)

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Hypoplastic or aplastic anemia can be elicited in the bovine by feeding of trichloroethylene-extracted soybean oil meal (TCE-SOM) (1,2). Development and course of this anemia indicate that in this species the bone marrow is the primary site of action of the toxic agent. Among circulating derivatives of

bone marrow, the composition of thrombocytes has apparently not previously been studied in any hypoplastic anemia.

Calves with induced hypoplastic anemia provided sufficient blood for preparation of thrombocyte concentrates in which the presence of nucleic acids and the activity of some hydrolytic enzymes could be investigated. Study of the latter was selected because sensitive analytical methods could be adapted for this purpose and because their activity in leukocytes is affected in other types of blood dyscrasias(3).

Methods. Young female Holstein calves used for other purposes(2) served as blood donors. Normal control calves were fed

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alfalfa, hexane-extracted soybean oil meal and milk while the others were rendered thrombocytopenic by feeding of TCESOM. A. *Preparation of thrombocyte concentrates*. All glassware which came in contact with blood was silicone-coated with Desicote.[†] Blood was collected at slaughter, by exsanguination, in an equal volume of anticoagulant solution (25 g sodium citrate, 9 g sodium chloride, 1000 ml distilled water). This mixture was centrifuged at 900 g for 5-10 minutes, or until a distinct boundary of erythrocytes had just formed. The turbid supernatant containing thrombocytes was siphoned into another container and centrifuged at 2000 g for 20 minutes. The supernatant was discarded and the sediment suspended in anticoagulant solution and centrifuged at 50 g for 5-10 minutes. The thrombocyte-rich supernatant was removed and its leukocytes, erythrocytes and thrombocytes were counted. An arbitrary value of not more than one erythrocyte/5000 thrombocytes was chosen as the minimum standard of purity, and when the erythrocyte contamination exceeded this number, the process of centrifugation at 50 g and removal of supernatant was repeated until the desired degree of purity was attained. Contamination from erythrocytes always exceeded that from leukocytes. The final thrombocyte concentrates were kept at -20°C until analyzed in duplicate. B. *Phosphatase determination*. The phosphatase method was adapted from that of Valentine and Beck(4). To release the enzyme, one volume of thrombocyte concentrate was treated for 30 minutes with 2 volumes of aqueous 2% saponin. To find the optimum pH for enzyme activity, substrates were prepared in the range from pH 2 to 11 by dissolving 0.5 g sodium 2-glycerophosphate and 0.42 g sodium diethylbarbital in water, adjusting the pH with 1 N acetic acid, citric acid or sodium hydroxide, and diluting to 100 ml with water. A mixture of 2.25 ml of substrate and 0.05 ml of 0.05 M MgCl₂ solution was warmed 10 minutes at 37°C, and 0.20 ml of thrombocyte-saponin mixture was added. After incubation at 37°C for 60 minutes, the phosphatase

was inactivated by addition of 0.5 ml of 30% trichloroacetic acid. In control experiments trichloroacetic acid was added to the substrate before addition of the thrombocyte-saponin mixture. Inorganic P was determined in 2.50 ml of clear, centrifuged supernatant by the method of Berenblum and Chain(5). Phosphatase activity was expressed as micro-moles of inorganic P liberated/hour/10¹⁰ thrombocytes. C. *Beta-D-glucuronidase determination*. This was done by adaption of the method of Fishman *et al.*(6). A mixture of 0.1 ml of thrombocyte concentrate, 0.8 ml of 0.1 M acetate buffer and 0.1 ml of 0.01 M phenolphthalein monoglucosiduronic acid[‡] was incubated at 37°C for 24 hours. Then 1 ml of 10% trichloroacetic acid and 2.5 ml of an alkaline reagent mixture (4 volumes of 0.4 M glycine buffer, pH = 10.45, plus 1 volume of 1.0 N NaOH) were added, the volume was adjusted to 5 ml, and the suspension centrifuged. The clear supernatant was decanted and its absorbancy read in a Beckman spectrophotometer at 555 mμ. Tubes containing only thrombocyte concentrate and buffer served as controls. For determination of optimum pH of beta-glucuronidase activity, a series of acetate buffers (pH 3.4-5.4) was prepared. After addition of trichloroacetic acid, calculated amounts of 0.04 M NaOH or 0.04 M acetic acid were added to adjust the pH, before development of the color, to that obtained when the mixture contained acetate buffer of pH 4.5. D. *Arylsulfatase determination*. The method was adapted from that of Rutenberg *et al.*(7) for histochemical demonstration of arylsulfatase. One ml of dilute (1:3) thrombocyte concentrate was incubated 24 hours at 37°C with 1 ml of substrate (25 mg 6-benzoyl-2-naphthyl-sulfate[§] in 80 ml hot aqueous 0.85% NaCl and 20 ml of 0.5 M acetate, pH 5.7). Then 0.25 ml of tetrazotized diorthoanisidine (100 mg in 100 ml 0.05 M phosphate buffer pH 7.6) and 3 minutes later 0.25 ml 40% trichloroacetic acid were added. The mixture was shaken vigorously with 5 ml of chloroform and centrifuged.

[†] Prepared from cinchonidine salt according to Fishman *et al.*(6).

[§] Obtained from Dajac Laboratories, Leominster, Mass.

[‡] Desicote, Beckman Instruments, Inc., Pasadena, Cal.

The chloroform layer was drawn off, shaken with a little anhydrous sodium sulfate, centrifuged and the absorbancy of the clear supernatant was read in the Beckman spectrophotometer at 540 $m\mu$ against standards prepared from 6-benzoyl-2-naphthol. Blanks were prepared by addition of thrombocyte suspension after that of trichloroacetic acid. E. *Esterase determinations*. Aliesterase activities were determined by adaption of the method of Hardin *et al.*(3). Substrates of 2-naphthyl acetate, 2-naphthyl laurate and 2-naphthyl stearate were synthesized(8). Buffer-substrate solutions were prepared immediately before use as follows: 2-naphthyl acetate: 0.5 ml of 2-naphthyl acetate solution (0.5% in acetone) was added with stirring to 17.5 ml of 0.05 M tris-(hydroxymethyl)aminomethane and 2 ml of water; 2-naphthyl laurate and 2-naphthyl stearate: 1 ml of solution (2.0% 2-naphthyl laurate in acetone, or 0.2% 2-naphthyl stearate in acetone), was added with stirring to 19 ml of 0.05 M tris(hydroxymethyl)aminomethane. For determination of aliesterase activity 1 ml of buffer-substrate, warmed to 37°C for 5 minutes, was mixed with 1 ml of diluted thrombocyte concentrate (diluted 1:15 when 2-naphthyl acetate was used, or diluted 1:4 with the other esters) and incubated 30 minutes (2-naphthyl acetate) or 2 hours (2-naphthyl laurate and stearate). Then, 0.25 ml of 0.4% tetrazotized diorthanisidine was added to all mixtures, and 3 minutes later, 0.25 ml of 40% trichloroacetic acid. The azo dye was extracted by vigorous shaking with 6 ml of chloroform, which was removed after centrifugation and clarified by shaking with a little anhydrous sodium sulfate. The absorbancy of the clear chloroform solution was read at 540 $m\mu$. In the blanks the thrombocyte suspension was added after the trichloroacetic acid. Aliesterase activity was expressed as μ moles of 2-naphthol liberated/ 10^{10} thrombocytes/hour. The optimum pH for each of the substrates was determined with buffers covering the range of pH 5 to 9. Acetylcholinesterase activity was determined by the method of Glick(9), using an incubation period of 24 hours. Activity was calculated in terms of μ moles of acetic acid liberated/24 hours/ 10^{10} thrombocytes. Pseudo-

TABLE I. Enzyme Activities of Bovine Thrombocyte Concentrates.

Enzyme	Range and mean $\pm$ S.E.	
	Normal calves*	Thrombocytopenic calves†
Acid phosphatase‡	.87-1.61 1.29 $\pm$.083	.77-1.64 1.23 $\pm$.071
Beta-D-glucuronidase§	12.2-27.7 20.2 $\pm$ 1.45	8.8-34.2 19.8 $\pm$ 1.86
Arylsulfatase	1.2-5.2 2.9 $\pm$ 1.4	1.4-4.6 2.6 $\pm$.3
Aliesterase		
2-naphthyl acetate	10.2-22.0 17.2 $\pm$ 1.20	5.1-49.1 25.1 $\pm$ 3.68
" laurate	.34-3.90 1.14 $\pm$.36	.40-3.20 1.74 $\pm$.21
" stearate	.08-.44 .22 $\pm$.04	.08-1.70 .55 $\pm$.12
Acetylcholinesterase**	10.2-49.2 24.4 $\pm$ 4.7	7.2-40.4 28.2 $\pm$ 3.0

* Values from 9 calves fed hexane-extracted soybean oil meal. Mean blood thrombocyte count was 664,000 $\pm$ 30,000/mm³.

† Values from 17 calves rendered thrombocytopenic by feeding of trichloroethylene-extracted soybean oil meal. Mean blood thrombocyte count was 269,000 $\pm$ 37,800/mm³.

‡ μ moles of inorganic phosphorus formed/ 10^{10} thrombocytes/hr.

§ μ moles of phenolphthalein formed/ 10^{10} thrombocytes/hr.

|| $m\mu$ moles of 6-benzoyl-2-naphthol formed/ 10^{10} thrombocytes/hr.

¶ μ moles of 2-naphthol formed/ 10^{10} thrombocytes/hr.

** μ moles of acetic acid liberated/ 10^{10} thrombocytes/24 hr.

cholinesterase activity was measured by modification of the method by Kalow and Lindsay (10). Two ml of benzoylcholine chloride solution (2.44 mg in 100 ml of M/15 phosphate buffer, pH = 7.4) was warmed to 37°C for 5 minutes, 0.5 ml of thrombocyte concentrate (diluted 1:10 with M/15 phosphate buffer, pH = 7.4) was added and the mixture incubated 30 minutes. Then, 1.7 ml of 1 M HClO₄ was added and after centrifugation, the absorbancy of the supernatant was read at 240 $m\mu$. Control analyses were made simultaneously in which the thrombocytes were added after the HClO₄. F. *Nucleic acids*. Thrombocyte concentrates were examined for deoxypentosenucleic acid by ultraviolet light absorption of perchloric acid extracts(11), deoxypentosenucleic acid P analyses, the indole reaction(12), and the Feulgen reaction made on smears(13). The presence of the pentose-

nucleic acid was investigated by ultraviolet light absorption of perchloric acid extracts (11), pentosenucleic acid P analyses, the orcinol reaction(14), and the methyl greenpyronin and ribonuclease method on smears(13).

Results. Table I is a summary of results of determinations of enzyme activities. As indicated by the relatively large standard errors of the means, the enzyme activities of thrombocytes from different normal or thrombocytopenic individuals varied greatly. There was no statistically significant difference, as determined by the "t" test(15), of the means of the 2 groups of animals and, among the thrombocytopenic specimens no correlation between the degree of thrombocytopenia and enzyme activity.

Phosphatase was most active at pH 5.0, a value similar to those reported for phosphatase in equine(16), rabbit(17) and human (16,18,19) thrombocytes. In contrast to Salvidio's(19) findings with human thrombocytes no alkaline phosphatase could be detected.

Bovine thrombocytes contained a beta-D-glucuronidase with optimum pH of 4.5. With the same substrate, Fishman(6) found no difference in beta-glucuronidase activities of thrombocyte-rich and thrombocyte-poor plasma and concluded, in contrast to Alexander (18), that human thrombocytes did not contain beta-glucuronidase activity. Arylsulfatase had a relatively low activity and an optimum pH of 5.7.

Recent work indicates that tissues of the rat(20), liver of the ox(21,22,23) and several tissues of man(24) contain arylsulfatases which can be differentiated through solubilities, affinities for various substances, and pH optima.

With the same substrate as used in these studies, Rutenberg *et al.*(7) could not detect arylsulfatase in any of the blood cells of the rat by a histochemical procedure. The pH optimum for aliesterase activity was 7.3 with 2-naphthylacetate as substrate and 6.9 with the other 2 esters and the activity decreased with increasing chain length of the acyl groups. Because of the lack of substrate specificity among esterases(25), the observed activities may reflect a difference in affinity of the same enzyme for various substrates or the

presence of more than one aliesterase in thrombocytes. In contrast to pancreatic lipase which is more active in the presence of 0.1 M sodium taurocholate(26), but like the esterase of rat liver(26), the aliesterase activity of bovine thrombocytes was inhibited by this reagent, to the extent of 83% with the acetate, 73% with the laurate and 58% with the stearate.

Our data confirm the recent report of Zajicek(27) of the occurrence of low cholinesterase activity in normal bovine thrombocytes. Moreover, both cholinesterase and aliesterase activity were inhibited to the extent of about 50% when, for other purposes, 6 calves were injected intramuscularly with 1.4 to 5.6 mg diisopropylphosphorofluoridate//100 kg body weight 90 minutes before collection of the thrombocytes.

Nucleic acids. Measurements of ultraviolet absorption of nucleic acid P in specimens containing $2-3 \times 10^9$ thrombocytes from normal or thrombocytopenic calves indicated the presence of only traces of pentosenucleic acid or deoxypentosenucleic acid. Tests for these components by the orcinol and ribonuclease reactions or the indole and Feulgen reactions respectively gave negative results. This is in accord with those of Maupin(28) and of Bestetti *et al.*(29,30) and with the widely accepted theory(31) that thrombocytes represent cytoplasmic fragments of megakaryocytes in which deoxypentosenucleic acid is largely confined to the nuclear structure.

The results of others suggesting that human (32) or equine(33) thrombocytes contain appreciable amounts of nucleic acids could be due to specie difference or to presence of relatively large amounts of adenosinephosphate which Bestetti and Crosti(30) found in human thrombocytes and which could be associated with protein.

Summary. 1. Bovine thrombocyte concentrates contained acid phosphatase, beta-D-glucuronidase, arylsulfatase, aliesterase (active with three fatty acid esters of carbon chain lengths: C_2 , C_{12} and C_{18}) and acetylcholinesterase but no alkaline phosphatase. Benzoylcholine was not hydrolyzed by throm-

|| Generously supplied by Merck, Sharp and Dohme Research Laboratories, West Point, Pa.

bocytes. 2. There was no significant difference in these enzyme activities of thrombocytes from normal or thrombocytopenic calves calculated in terms of activity per 10^{10} thrombocytes. 3. There was no significant quantity of deoxypentose nucleic or pentose nucleic acid in bovine thrombocyte concentrates from either normal or thrombocytopenic calves. 4. Thrombocytes released by hypoplastic bonemarrow could not be differentiated from normal thrombocytes by the tests used.

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Relationship of Blood Heparin Levels to Serum Lipoproteins and Cholesterol Levels in Fasting Subjects.* (23936)

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In recent years much of the investigative effort on atherosclerosis has centered about

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studies of lipid metabolism. Since Hahn demonstrated that parenterally-administered heparin had an *in vivo* clearing action upon lipemic blood(1), numerous investigations have been made concerning the nature of this clearing action. Anderson showed that turbidity of lipemic plasma could be cleared *in vitro* by mixing lipemic plasma obtained prior to heparinization with that obtained 5 minutes after heparinization(2). Graham *et al.* demonstrated that heparin administration was followed by decrease in concentration of low-density lipoproteins of high S_r rate together with increase in concentration in low density lipoproteins of low S_r rates(3). The mechanism of the heparin-induced clearing and lipoprotein transformations was shown by Shore *et al.* to be associated with activation of a lipolytic enzyme acting on triglycerides(4). All these observations have been abundantly confirmed. Ability of parenterally-administered heparin to produce such striking effects upon lipids has naturally raised the question of possible relationship of spontaneously-occurring blood heparin to blood lipid and lipoprotein levels. Methods of blood heparin measurement have been reported by Jaques, Mitford and Richer, Jaques, Morehouse and Steward, Monkhouse and Jaques, Bassiouni and Gibson(5,6,7,8,9). Direct investigation of relationships between blood lipids and blood heparin levels have recently been made by Oshima and his associates in Japan. Oshima and associates and Murakami and associates studied the relationship of electrophoretically-determined β/α -lipoprotein ratios with blood heparin levels and concluded that a negative correlation exists between these variables(10,11). Further, Oshima *et al.* have reported a negative correlation between blood heparin level and ultracentrifugally determined S_r^0 0-12 and S_r^0 12-20 lipoprotein levels, respectively. No significant relationship was found by these workers between blood heparin levels and those of S_r^0 20-100 lipoproteins(10).§

It is of interest to know whether or not similar relationships of blood heparin level to blood lipids exist in various "races" of man because of variations in diet and mode of life.

Further, confirmation of the general findings described is of value. The present study was designed to determine extent of relationship between blood heparin and blood lipid levels in a sample of males in the United States.

Materials and methods.|| *Subjects.* 105 male schizophrenic patients, between 40 and 50 years of age, from Stockton State Hospital served as subjects. Blood samples for both heparin and lipid determinations were obtained from fasting subjects. The choice of schizophrenic patients for such a study may be questioned. However, with respect to blood lipid levels, there exists excellent evidence to indicate that no significant difference in blood lipid levels exists between such subjects and those of the population-at-large(12). *Heparin determination.* A modification of the Bassiouni method for determination of heparin (and heparin-like substances) of blood was employed. The modifications introduced provided excellent reproducibility in the heparin assay. *Preparation of samples.* Deliver 10 ml of blood directly into tube containing 5 ml of 3.8% sodium citrate solution and mix. Three ml of this mixture is used for the determination. The blood-citrate mixture is centrifuged for 10 minutes at 1500 rpm in a clinical centrifuge (International Type II) and then the supernatant (plasma-citrate mixture) is pipetted off into a glass-stoppered 25 ml graduated test tube. To the residue, containing blood cellular elements, is added 2 ml of physiological saline solution, tube contents mixed well, and centrifuged as above. The supernatant is pipetted off and combined with bulk of the plasma. This combined plasma sample is made up to 6 ml with physiological saline. The final 6 ml represents the plasma content of 2 ml of blood. *Extraction of Heparin.*¶ To this 6 ml is added 1 ml of 2 N sodium hydroxide, tube is stoppered, and contents mixed well. Then tube is heated in 50°C water bath 40 minutes. Next, with

|| Since heparin and lipid determinations were performed separately and without knowledge of the corresponding findings, this study is essentially a "blind study."

¶ Designation "heparin" includes true heparin and heparin-like substances of blood following heparin through this chemical procedure.

§ These studies were performed on fasting subjects.

tube still warm, 5.5 g of ammonium sulfate crystals are added, the mixture shaken vigorously, and returned to 50°C water bath for 20 minutes. Temperature is then rapidly increased to 75°C in boiling water. After vigorous shaking, mixture is filtered through sintered-glass, yielding approximately 8-9 ml of filtrate. The residue is washed with 3 ml of saturated ammonium sulfate solution, filtered, and filtrate combined with original filtrate. Distilled water is added to make up to 23 ml. Pressure is then gradually reduced by pumping until no gas bubbles appear even with vigorous tube tapping. Distilled water is added to make up to 23 ml. *Dyeing, precipitation and colorimetry.* 0.2 ml of 0.1% dimethyl thionine solution is added to 15 ml of filtrate, the combined solution mixed and stored 48 hours at room temperature in the dark. The precipitate formed is centrifuged at 3000 rpm for 40-60 minutes and the supernatant decanted. The precipitate is washed with 2 ml of 90% saturated ammonium sulfate solution and re-centrifuged at 3000 rpm for 30-40 minutes. After decanting supernatant, 4 ml of buffer solution (1 vol. acetone mixed with 2 vol. 0.1 N glycine in 0.4 N sodium hydroxide) is added to dissolve precipitate. After dissolving, tube is spun in clinical centrifuge and the supernatant decanted and read within 30 minutes at 526 $m\mu$ in a spectrophotometer (Beckman). *Blank.* To 11.5 ml of saturated ammonium sulfate solution is added 1 ml of 2 N sodium hydroxide and distilled water to make 23 ml. This mixture is heated to 75°C and treated like the plasma samples. *Calibration.* Solutions of 0.01, 0.02, 0.05, 0.10 and 0.15 mg/ml of heparin (calcium heparin, 105 units/mg from Southern California Gland Co.) are each mixed with 1 ml of 2 N sodium hydroxide, 11.5 ml of saturated ammonium sulfate, and distilled water to make up to 23 ml, and then treated like the blanks. Recovery of heparin added to plasma in 6 cases averaged 88%, utilizing 0.05 mg of heparin per sample. *Reproducibility.* Reproducibility, tested on 28 pairs of blind duplicates, showed a technical error of less than 5% (0.08 mg/100 ml for a mean level of 2.58 mg/100 ml). Our method yields experimental values of blood "heparin" larger than anticipated from

TABLE I. Mean Values and Correlations of Variables Studied (105 Cases*).

Variable	Mean and stand. dev., mg/100 ml of serum	Correlation with blood "Heparin," r (Pearson)	Significance test for r
"Heparin"	3.07 $\pm$ 1.12†		
S _f ^o 0-12	379.3 $\pm$ 92.7	-.49	p < .001
S _f ^o 12-20	47.6 $\pm$ 24.4	-.34	"
S _f ^o 20-100	80.8 $\pm$ 60.5	-.01	N.S.
S _f ^o 100-400	40.2 $\pm$ 8.3	+.13	"
Cholesterol	242.6 $\pm$ 47.6	-.47	p < .001
HDL ₁	24.8 $\pm$ 8.7	-.01	N.S.
HDL ₂	62.4 $\pm$ 52.2	.00	"
HDL ₃	210.5 $\pm$ 39.5	-.13	"

* All variables were studied in 105 cases except the 3 high density lipoproteins, HDL₁, HDL₂ and HDL₃, which were studied in 99 cases.

† mg/100 ml of blood rather than serum.

Bassiouni's original method, probably the result of a higher percentage recovery of "heparin." *Lipid determinations.* High and low density lipoproteins were determined by the method of de Lalla and Gofman(13) and serum cholesterol by that of Colman and MacPhee(14).

Results. In Table I are presented means and standard deviations for all variables measured and the Pearson correlation coefficient, r, between each lipid variable and blood "heparin." Significant inverse correlations were demonstrable for the following pairs of variables:

S _f ^o 0-12 vs heparin	r = -.49 (p < 0.001)
S _f ^o 12-20 vs heparin	r = -.34 "
Serum cholesterol vs heparin	r = -.47 "

Neither the S_f^o 20-100, S_f^o 100-400, nor any of the 3 high-density lipoproteins showed any provably significant relationship with blood "heparin" level. The relationship of serum cholesterol with blood "heparin" is undoubtedly a reflection of corresponding relationship of S_f^o 0-12 and S_f^o 12-20 lipoproteins with "heparin." These lipoproteins represent the major cholesterol-bearers of blood.

While pharmacologic studies with parenteral heparin indicate that lipoproteins of S_f^o 20 and higher are those largely affected by heparin-activated lipase, this study shows no quantitative relationship between blood "he-

parin" and such lipoproteins. On the other hand, pharmacologic studies *in vivo* and *in vitro* indicate low-density lipoproteins of S_f° 20 and lower may be a product of action of this lipase and this study shows an appreciable inverse correlation of levels of S_f° 0-20 lipoproteins with blood "heparin." None of the high density lipoproteins (HDL₁, HDL₂ or HDL₃) show quantitative relationship to blood "heparin." While mechanism remains obscure, it does appear that levels of spontaneously occurring heparin-like substances are quantitatively related to levels of certain of the blood lipoproteins.

Discussion. These findings on fasting blood from a sample of U.S. males are consistent with those of Oshima *et al.* on fasting blood from a Japanese population sample. Engelberg, studying non-fasting bloods of a mixed group of subjects, preponderantly atherosclerotics, reported a very low order inverse correlation of S_f° 0-12 lipoproteins with blood heparin levels(15). The population sample studied, the heparin method, and the use of non-fasting subjects all render it difficult to compare his results with those reported here. However, it is of interest that Engelberg's study and this one point to an inverse relationship of S_f° 0-12 lipoproteins with blood heparin levels.

Summary. 1. A highly reproducible modification of the Bassiouni method for determination of spontaneously-occurring blood heparin-like substances has been described and evaluated. 2. A mean level of 3.07 mg/100 ml of blood heparin-like substances was determined for a sample of 105 fasting U.S. schizophrenic males 40 to 50 years old. This sample group showed lipoprotein and cholesterol values very close to those characteristic

for other samples of U.S. males previously studied. 3. Highly significant negative correlations were demonstrated for the following pairs of variables, S_f° 0-12 lipoproteins versus blood "heparin," S_f° 12-20 lipoproteins versus blood "heparin," and serum cholesterol versus blood "heparin."

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Comparative Study of Uptake and Distribution of Hg^{203} Given as Labelled Chlormerodrin (Neohydrin).*† (23937)

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It is well established that organic mercurial compounds are preferentially concentrated in renal tissue of fish(1), rat(2), rabbit(3), dog (2) and man(4). The present investigation provides quantitative information as to uptake and distribution of organic mercurial diuretic by a variety of tissues in poikilothermic animals such as amphibia, reptiles and fish, and a comparison is made with higher homoiothermic forms including chicken, rat and dog. As far as renal uptake was concerned, attention was particularly directed to site of preferential mercury uptake within the nephron and to species differences regarding time course of mercury binding and maximum concentration attained.

Methods. A total of 40 experiments was performed on specimens of mudpuppies (*Necturus maculosus*), bullfrogs (*Rana catesbeiana*), turtles (*Terrapene carolina*), carp (*Cyprina carpio*), chickens and mongrel dogs. Each animal was injected intramuscularly with a neutralized solution of Hg^{203} -labelled chlormerodrin (3-chloromercuri-2-methoxypropylurea, Neohydrin). In mudpuppies, frogs, turtles, fish and chickens, a site was chosen for injection which was not predominantly within the drainage area of the portal circulation. At time intervals ranging from 2-70 hrs after injection, the animals were anaesthetized as follows: 30 mg Nembutal/kg (dogs), 1.5% Urethan (Necturi), pithed (bullfrogs) or stunned (carp, chicken). Blood was obtained either from the heart (turtle, bullfrog), aorta (mudpuppies), carotids (chicken), postcaval vein (carp) or femoral artery (dogs). Samples of kidney, liver, spleen, heart muscle, gut, abdominal wall muscle, lung and gills were removed immediately following collection of blood, and adherent connective tissue removed by dissection. In

Necturus separation of kidney into a medial and lateral section was undertaken since anatomical evidence(5,6,7) indicates that the lateral part of kidney of Necturus is made up predominantly of proximal convoluted tubules and collecting ducts (ureteral tissue having been removed by dissection) while the medial strip of kidney contains the following parts of the nephron: glomerulus, distal tubular segment, neck, intermediate tubule and nephrostome. Separation into medial and lateral strip of tissue was done by sagittal dissection about 1 mm lateral to the visible row of glomeruli. Samples of tissues were blotted, weighed on torsion-balance, transferred to standard test tubes and counted in well-type scintillation counter. Plasma and tissue concentration of the organic mercurial compound are expressed in terms of μg mercury/g wet tissue.

Results. In Table I data obtained in Necturus are summarized. It is evident that, with one exception, the concentration of radiomercury in renal tissue exceeds that of plasma. Furthermore, a comparison of the respective tissue/plasma concentration ratios (T/P ratios) shows that concentration of Hg^{203} , given as radiochlormerodrin, in lateral kidney tissue was always higher than that taken up by medial strips of the kidney. Accordingly, tissue/plasma concentration ratios were consistently higher for that part of kidney which constitutes predominantly proximal convoluted tubules. It can also be seen that, while there is some scatter, there is a trend toward increased uptake of mercury with time elapsed after administration. Thus a comparison of Exp. No. 2 and 9, or 3 and 8 shows considerably higher concentrations of radiomercury within the renal tissue, for a comparable dose, 26 hrs or later after radiochlormerodrin had been given.

Tissue/plasma ratios for Hg^{203} exceeded one in only liver, gut and, occasionally, spleen (range of T/P ratios: liver 1.13-21.6, gut: 0.33-4.17, spleen: 0.40-2.15). Of these tis-

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TABLE I. Plasma and Kidney Concentration and Tissue/Plasma Concentration Ratios of Mercury²⁰³ Given as Radiochlormerodrin.

Exp. No.	Dose of Hg ²⁰³ , mg/kg	Time after inj., hr	Hg conc. (γ/g)			T/P ratio	
			Plasma	Kidney Lateral	Medial	Lateral	Medial
2	12.7	2	8.4	13.2	7.3	1.6	.9
3	9.2	2	4.1	8.0	2.2	2.0	.6
1	15.0	3	17.7	10.7	8.7	.6	.5
4	9.7	8	10.2	86.2	44.4	8.4	4.3
5	5.8	16	12.2	106.0	52.5	8.7	4.3
6	5.0	16	4.2	50.2	36.1	12.0	8.6
12	1.0	19	.9	8.3	.6	9.2	.7
13	1.0	19	.6	7.1	3.6	11.8	6.0
7	6.3	26	8.6	106.8	62.6	12.4	7.3
8	8.8	26	7.3	251.4	115.6	34.5	15.9
10	2.8	46	6.9	261.2	40.0	38.2	5.9
11	2.5	46	1.67	36.7	13.8	22.0	8.3
9	12.1	70	5.3	153.0	87.0	29.0	16.4

sues, liver consistently bound more mercury than did gut or spleen. All other specimens studied (heart muscle, skeletal muscle and gills) showed T/P ratios below one, and skeletal muscle always exhibited the lowest mercury concentration.

Table II shows that in all other species studied a considerable degree of Hg²⁰³ concentration occurred in the kidney. In both poikilothermic and homoiothermic animals mercury storage occurred in the kidney, for a

high mercury content is sustained despite a progressive fall in plasma concentration with time. In our series of poikilothermic animals, the turtle had both the highest T/P ratios and the highest mercury content in renal tissue.

No preferential binding of mercury by liver, gut and spleen was observed in turtle and frog (range of T/P ratios: frog: liver 0.14-0.42, gut 0.02-0.62, spleen 0.17-0.92; turtle: liver 0.30-1.04, spleen 0.17-0.38, gut 0.06-0.48). In contrast these concentration

TABLE II. Plasma and Kidney Concentrations and Tissue/Plasma Concentration Ratios of Mercury²⁰³ Given as Radiochlormerodrin in Poikilothermic and Homoiothermic Animals. Data for rats and 2 dogs are taken from Borghgraef, R. R. M. and Pitts, R. F. (2).

Time after inj., hr	Hg ²⁰³ concentration								
	Kidney γ/g	Plasma γ/g	T/P ratio	Kidney γ/g	Plasma γ/g	T/P ratio	Kidney γ/g	Plasma γ/g	T/P ratio
Carp									
5	32.4	1.6	20.8	111.6	9.7	11.5	80.0	6.2	12.9
5	10.5	.2	52.5	138.0	11.9	11.6	111.0	6.1	18.2
18-24	24.9	1.4	17.8	100.0	5.6	17.9	129.0	2.7	47.8
"	32.3	4.1	7.9	121.4	7.9	15.4	203.0	3.9	52.1
46-48	18.6	.3	62.0	75.0	3.6	20.8	233.0	2.7	86.3
"	16.0	.6	26.7	136.9	6.4	21.4	265.0	3.4	78.0
Chicken									
1-2				55.6	4.2	13.3	163.0	.9	181.0
"				75.2	.8	94.2	131.0	1.0	131.0
3-6	32.8	.4	82.0	97.7	.6	163.0	74.5	.8	93.2
4-6	34.2	.2	171.0	108.0	.2	540.0	83.3	.22	378.
12				94.0	.2	470.0			
18-24	16.2	.2	81.0	97.5	.1	975.0	13.3	.01	1330.
"	19.2	.2	96.0						
46-48	4.2	.06	70.0						
"	23.9	.1	239.0						

Dose of Hg in all cases from 1.0 to 2.0 mg/kg body wt.

* Hg administered intrav.

† Kidney cortex only.

ratios were often above one in carp (range of T/P ratios: liver 0.76-5.0, spleen 0.76-5.14, gut 0.26-1.84). In all animals skeletal muscle again contained the least mercury.

The lower section of Table II contains results on renal mercury uptake in the group of homoiothermic animals studied. Data obtained on rats and 2 dogs by other authors(2), using the same mercurial compound, have been included for comparison. It is evident that in all animals tissue plasma concentration ratios exceeded those obtained in the poikilothermic group. This, however, is a reflection of low plasma concentration ratios rather than an increased accumulation of Hg^{203} in the kidney. With the exception of the dog, in all animals there was a slow fall in kidney radiomercury content over the time period studied.

Discussion. The results presented indicate that radiochlormerodrin is bound to a considerable degree by kidney tissue in a variety of animals other than mammals. While in general renal storage of mercury is sustained up to 46-70 hrs, our findings show significant species differences as to absolute amount of mercury bound and time course of renal mercury accumulation. Thus in *Necturus* and turtle, maximum concentration was reached more slowly. The delay in reaching a high concentration of radiomercury in the kidney of *Necturus* may account for failure to obtain a significant diuresis in short-term experiments(8). Since the nature of binding of mercury in renal tissue is poorly understood, nothing can be said about the cause of the observed species differences. Our results on regional distribution of Hg^{203} within

the kidney indicate that it is the proximal tubule which preferentially binds mercury. This region is most likely also the site of secretion into urine since in the aglomerular toadfish whose nephron consists of proximal tubules only, radiomercury has been found in urine in concentrations higher than in plasma(1).

Summary. The uptake and distribution of Hg^{203} given as labelled chlormerodrin (Neohydrin) has been studied in the mudpuppy, carp, bullfrog, turtle, chicken and dog. Preferential binding of radiomercury by renal tissue was found in all animals with most tissue/plasma ratios of Hg^{203} well above one. For a given dose poikilothermic animals showed a slower accumulation and loss of radiomercury than did homoiothermic specimens. With the exception of the dog, in all animals there was only a slow fall in renal content of radiomercury over a time period of 46 to 70 hrs. In *Necturus* maximum mercury uptake was found in a part of the kidney made up predominantly of proximal tubules.

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Preliminary Observations on Bone Isografts within Diffusion Chambers.* (23938)

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A recent historical review(1) of autogenous and homogenous bone grafts indicates real uncertainty as to the origin of newly formed bone and cartilage around the graft. Most investigators favor the concept that new bone arises from surrounding host connective tissue rather than from surviving cells of the graft. Some proponents of this "metaplasia theory" believe that *all* elements of a bone transplant die and are replaced by host tissue—even in *autogenous* bone transplants(2). Indirect evidence, however, supports the hypothesis that new bone is formed by living cells of the graft. Ham and Gordon(3) transplanted bone chips into muscle following a multiple freezing and thawing procedure designed to kill bone cells. No new bone was found adjacent to these chips as compared with untreated bone chips, indicating that new bone was derived from surviving osteogenic cells rather than from host connective tissue. Experiments in our laboratory utilizing fresh and autoclaved young mouse calvaria implanted subcutaneously into 6-week-old mice have yielded similar results (unpublished data). Although both experiments confirm the necessity for utilizing fresh, untreated bone to obtain new bone formation in these sites, neither experiment provides direct proof as to origin of new bone-forming cells. It is possible that *living* cells of the implant "induce" host cells to become osteogenic. In view of difficulties inherent in interpretation of histological evidence presented heretofore, we have attempted a different approach to this problem, whereby it is possible to "partition off" living implants within "diffusion chambers"(4) which allow diffusion of nutrients and metabolites through a porous filter between host tissue and implant *without the*

passage of cells. Under these conditions, new bone formation could conceivably occur in the following locations: 1) *Inside chamber exclusively*, indicating that surviving cells of implant are capable of osteogenesis, whereas metaplasia of host connective tissue does not occur. 2) *Outside chamber exclusively*, indicating that although the implant does not retain the capacity to form bone, it is capable of exerting an effect across the filter on surrounding host connective tissue, resulting in differentiation of bone-forming cells. 3) *Inside and outside the chamber*, indicating that newly formed bone occurs as result of osteogenesis of cells of implant plus those of host.

Material and methods. Calvaria of 2 to 3-day-old Swiss albino mice of inbred Webster strain were removed aseptically, placed in Petri dish containing 100 units each of penicillin and streptomycin/ml of Gey's Balanced Salt Solution and divided in half along the median suture. Each half was transferred to a diffusion chamber which had been partially prepared by gluing a Millipore Filter‡ to one side of a thin plastic ring. After insertion of the fragment into the open chamber, it was sealed by attaching a second filter to the open side of the ring. To minimize chamber breakage (which occurred frequently during initial experiments) 2 modifications were made: 1) plastic rings, 1.5 mm thick and center hole 12.5 mm in diameter, were made with a wide flange (4.5 mm) to allow greater surface area for gluing, and 2) stronger, nylon-reinforced Millipore Microweb Filters (19 mm in diameter, 150 μ thick and 0.45 μ pore size) were utilized. Six-week-old mice of same strain as donor tissue were utilized as hosts for chamber implants. Following anesthetization with sodium pentobarbital, an incision was made along right dorsal side of each animal. The skin was separated from underlying muscle with blunt dissection and a pouch created for

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† U.S.P.H.S., Senior Research Fellow.

‡ Obtained from Millipore Filter Corp., Watertown, Mass.

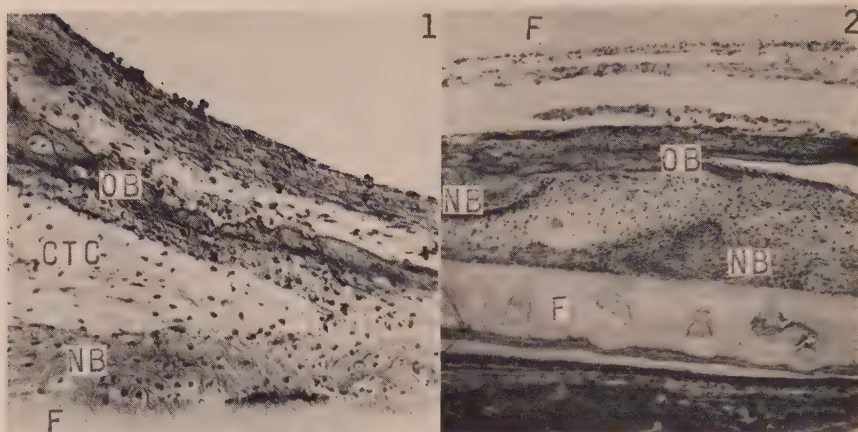


FIG. 1 ($\times 100$). 4-wk chamber implant showing original curved bone of calvarium within chamber (one filter removed). Note loose connective tissue cells which migrated from implant and osteo-chondroidal tissue on inner surface of filter. Symbols: F = filter, OB = original bone, NB = new bone, OC = osteo-chondroid, CTC = connective tissue cells.

FIG. 2 ($\times 60$). 8-wk chamber-implant with both filters in place. The thin original bone is covered in several areas by new bone. In addition, new bone has been laid down on inner surface of filter. "Holes" within lower filter are cross-sections of nylon reenforcement.

reception of diffusion chamber. After insertion of chamber, the incision was closed with nickel-silver wound clips. The animals were sacrificed at intervals of 2, 4 or 8 weeks following implantation, then the chambers and adherent host tissues were removed and fixed overnight in 10% neutral formalin. Following fixation, the plastic rings were cut away prior to decalcification with formic acid and sodium citrate(5). During handling procedures leading to paraffin blocking, any filter not adherent to the tissue within the chamber would tend to separate from the specimen and pull away. However, breakage of the chamber *in vivo* was readily detectable histologically, usually as infiltrating host blood vessels. The following preliminary results are based on histological examination of hematoxylin and eosin stained slides of 20 successful chamber implants.

Results. Marked growth and proliferation of cells from the original transplant occurred within the chambers. Differentiation of cells into bone or bone-like tissue occurred as early as 2 weeks after implantation, and exclusively *within* all chambers on inner surface of filter, surface of original bone, and, occasionally, as scattered islands within the loose connective tissue.

Fig. 1 represents a 4-week implant showing original dark-staining curved bone of calvar-

ium within a chamber (one filter removed). Loose connective tissue cells have proliferated from implant, bridging the gap between original tissue and adherent filter. Cell differentiation has occurred on inner surface of filter, giving rise to new, bone-like tissue containing cartilage elements. Although cell migration through the filter was never noted, partial penetration of cytoplasmic extensions occurred frequently. In some areas a distinct, thin basophilic zone, (possibly an acid mucopolysaccharide) could be distinguished within the filter immediately adjacent to newly formed bone-like tissue.

Further organization of loose connective tissue was found in older, 8-week chambers, Fig. 2, (both filters in place). In several areas, the thin, original bone appears covered by new bone. In addition, new bone with occasional cartilage-cells may be seen on inner surface of filter.

Higher magnification of this area (Fig. 3) shows osteo-chondroidal type of tissue laid down on filter of a 4-week chamber implant. Old bone may be recognized readily in upper portion of photomicrograph by its dark-staining appearance and relatively poor cellularity. Fig. 4 shows the more compact, bone-like tissue found on filter of an 8-week chamber implant. Large cartilage-like cells are present within this tissue as well.

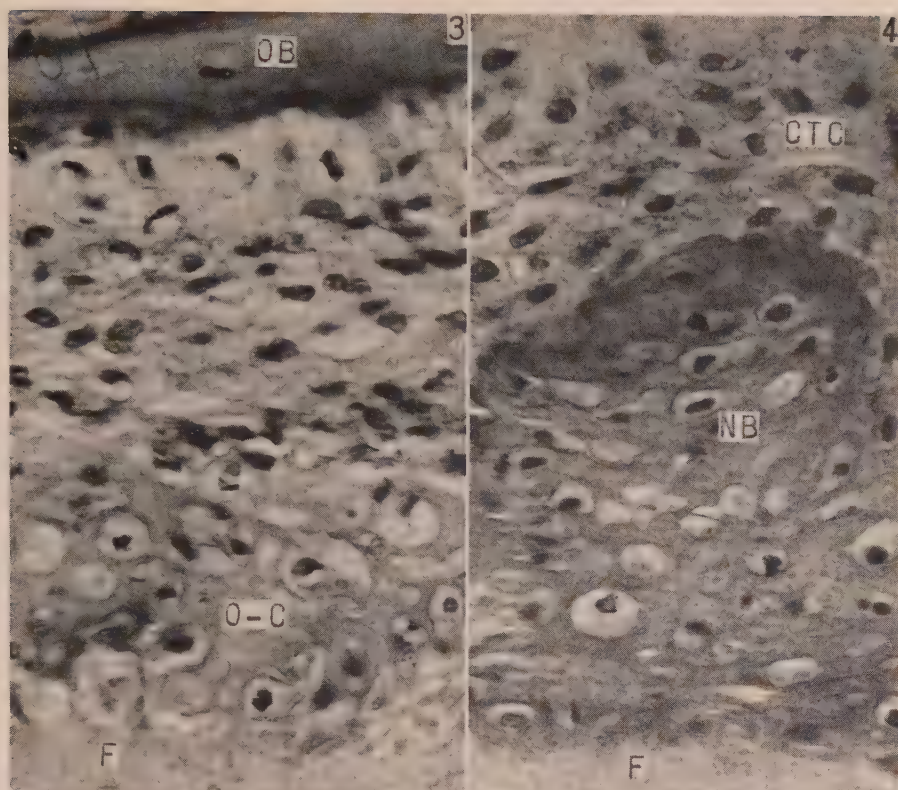


FIG. 3 ($\times 430$). Higher magnification of Fig. 1. Note osteo-chondroidal tissue laid down on inner surface of filter.

FIG. 4 ($\times 430$). Higher magnification of new bone on filter.

Newly differentiated tissue formed in relation to the original bone appeared somewhat more typical of young, active bone. Fig. 5 shows a 4-week chamber with new bone formed around cut edge of old, dead bone. Occasionally, such apposition of new bone filled the entire space between old bone and filter, as found in the 8-week chamber shown in Fig. 6.

Discussion. Our findings provide additional evidence that some cells of bone isografts not only survive, but show excellent ability to proliferate and form new bone and cartilage. Although failure to find any new bone or cartilage on the host side of intact filters may suggest that the "metaplasia theory" is untenable, the possibility cannot be excluded that induction may be responsible for some of the new bone found surrounding a fresh bone autograft in *direct physical contact* with host connective tissue. It should be noted that sepa-

ration of embryonic inductor and reacting tissue by thin artificial barriers has been shown to block induction *in vivo*. However, diffusible inductor substances can be clearly demonstrated *in vitro* (6). Grobstein (7), working with thin filters *in vitro* has shown that induction with various types of tissue can occur despite a cytoplasmic separation of $20\ \mu$. He suggests that induction may involve interaction between intercellular materials, such as proteins and complex polysaccharides which pass through the filter (8). In view of his findings, it is possible that maintaining chambers in animals for longer periods or using thinner filters might result in formation of bone on the outside of chambers through a mechanism involving bone induction.

It is of interest that "inductive" properties have been ascribed to bone homografts to explain their successful application in bone-healing studies. It has been suggested that bone

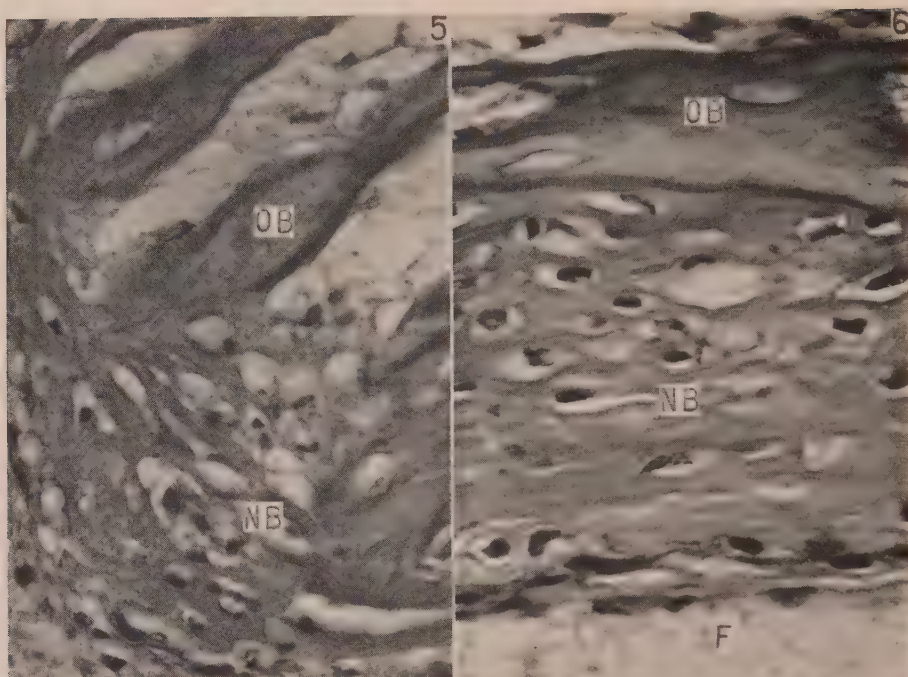


FIG. 5 ($\times 430$). 4-wk chamber-implant with new bone formed around cut edge of old, dead bone.

FIG. 6 ($\times 430$). 8-wk chamber-implant with apposition of new bone on surface of old bone. Note cytoplasmic extensions penetrating into filter.

homograft "provides a stabilizing scaffolding for ingrowing, undifferentiated, mesenchymal cells which, in turn, may be *induced* to become osteogenic by the physiochemical status of the very lattice upon which they grow(9)." Similarly, one might argue that the cellulose ester filters of our chambers have "inductive" properties in view of the new bone and cartilage laid down on their inner surfaces. However, failure of filter to induce host connective tissue to lay down bone and cartilage on the outside of the filters seems to preclude such a mechanism. In contrast to host connective tissue cells, the fibroblast-like cells migrating from original bone implant within the chamber have retained the ability to form bone and cartilage. These reversible changes in cell morphology and differentiation suggest that our findings might be regarded as examples of *cell modulation*(10) rather than induction.

Summary. 1. Surviving cells of bone isografts implanted subcutaneously within diffusion chambers form new bone and cartilage on the surface of original implant and on in-

ner surface of filter. 2. No new bone formation was observed on exterior of filter. 3. It is suggested that the results obtained within diffusion chambers are due to cell modulation rather than induction.

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Synergistic Reaction Between *p*-Phenylenediamine and Melanoma Components.* (23939)

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An unaccountable oxygen uptake has been observed when melanoma extracts or homogenates are tested manometrically with *p*-phenylenediamine (PPDA) (1-4). This compound is ordinarily employed as a cofactor in Warburg measurement of cytochrome oxidase activity in tissue slices or extracts, and reduces cytochrome c to the enzymatically oxidizable or usable form. Melanoma homogenates, in contrast to most other tissues, are unique in oxidizing added PPDA by an unknown mechanism other than the cyanide sensitive cytochrome system. Specifically, when PPDA is added to non-melanoma tissue extracts deprived of cytochrome c, or otherwise inactivated, no significant oxygen consumption occurs beyond the slight autooxidation of PPDA itself, whereas with extracts from malignant pigmented melanoma a significant additional oxygen uptake is found (1-3). These observations are illustrated in Fig. 1 and are further discussed with experimental results. In experiments to elucidate this phenomenon it was discovered that an accepted melanoma metabolite, dihydroxyphenylalanine (DOPA), was capable of combining at physiological temperatures with PPDA in a relatively vigorous oxygen consuming reaction when the 2 purified compounds were combined *in vitro*. A preponderance of the resulting products were pigmented and grossly resembled natural melanin (Fig. 5). A small amount of CO₂ was also formed. The following experiments illustrate some characteristics of the reaction, evidence for its relationship to the above melanoma phenomenon, and some possibilities for its exploitation in diagnosis and chemotherapy of

malignant melanoma.

Materials and methods. *p*-Phenylenediamine (PPDA), known also as Ursol and PPD, was received from several sources (Matheson, Coleman & Bell; Merck; Amend; and Eastman Kodak Co.). Although the autooxidative rates varied somewhat with source and purity (3), we noted no significant departures from the synergistic reaction reported here. Under some conditions certain differences are seen between the free base and the neutralized dihydrochloride; however, only the free base was employed in experiments of this report. 3,4 - Dihydroxyphenyl - DL-alanine (DOPA) was from Nutritional Biochemicals Corp. Its stock solution, as well as PPDA, was freshly prepared for each experiment. Oxygen consumption was measured in air by standard manometric procedures at 38°C with the vessels shaken at 112 cycles/minute and an amplitude of 4 cm. Reagent concentrations are expressed in molar terms and were calculated as final concentrations in the manometric vessel following tipping of sidearm component. Other pertinent details are given with the descriptions of specific experiments or are contained in the Figures.

Results. The experiment illustrated in Fig. 1 demonstrates several types of oxygen consumption when a cell-free Cloudman S91 mouse melanoma extract containing enzymically active melanin granules and the usual supernatant components(5) was tested manometrically under various conditions with and without PPDA. The upper 2 curves are persuasive in demonstrating that the reaction obtained when PPDA was added to melanoma extracts is probably not enzymatic in nature since the oxidation factor associated with the tumor is both KCN and heat insensitive. The inactivated fractions contained the same components as the nearly vertical curve depicting the cytochrome oxidase activity of the extract except for the

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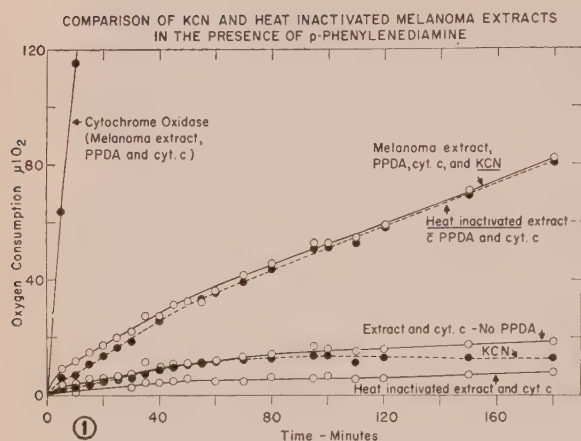
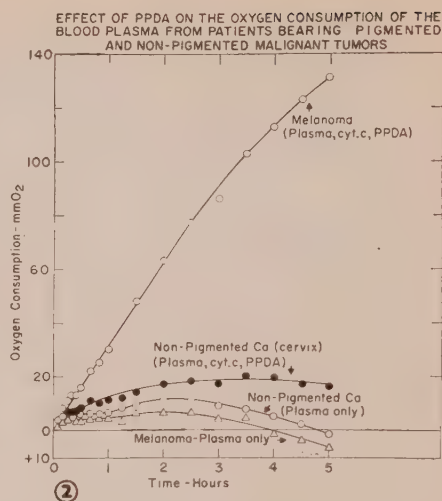


FIG. 1. Non-enzymic oxidation of *p*-phenylenediamine (PPDA) in KCN and heat inactivated mouse melanoma extract. (0.5 ml of 10% Cloudman S91 aqueous extract; phosphate buffer, 0.05 M, pH 7.4; cytochrome c, 5×10^{-5} M; PPDA, 0.05 M; final vessel volume, 2 ml.)

FIG. 2. Differential oxygen consumption of plasma from a melanoma patient compared with plasma from a patient with a non-melanotic malignancy. (Heparinized plasma; 0.02 M PPDA; 0.04 M phosphate buffer, pH 7.4; cytochrome c, 5×10^{-5} M but not essential.)



addition in one case of KCN to give a final vessel concentration of 0.003 M. The nearly identical broken line curve shows the similarity of oxygen uptake when the enzymes of the extract had been denatured by heating in boiling water for 10 minutes. The congruity of the two inactivation curves also indicates that essentially all oxidative enzyme activity in this melanoma extract is cyanide sensitive. The enzymatic dependence on an added cytochrome c reductant is shown by the lower endogenous oxidation curve where all necessary components for cytochrome oxidase activity are present except PPDA, or such equivalents as ascorbic acid or cytochrome c reductase. This particular oxidation-reduction state of melanoma tissue is probably related to the high differential PPDA response phenomenon described by Burk, *et al.* for this tumor(6). There is, of course, no significant oxygen consuming reaction between PPDA and cytochrome c at the concentrations usually employed in enzyme assays or in these experiments(3).

A reaction similar to that seen with melanoma extract has repeatedly been observed with the blood and urine of patients with advanced pigmented melanoma. Fig. 2 illustrates the differential oxygen consumption ob-

tained when PPDA is added to the plasma of a patient with disseminated pigmented melanoma as compared with a patient in a comparable stage of a non-melanotic malignancy. Although the identity of this PPDA reacting substance has not been established by isolation it seems permissible to assume that it is a metabolic product of the extensive pigmented metastases present in the patient. This reaction provides the basis for a possible diagnostic procedure and is being further studied. One of the difficulties to be overcome is the occasional presence in non-melanoma urines and plasmas of interfering substances that are capable of giving false positives.

The time-course curves of the oxygen consuming reaction when PPDA was added to DOPA and closely related compounds at pH 7.4 are shown in Fig. 3. It may be seen that the two ortho hydroxyl groups are essential for the reaction since neither tyrosine nor phenylalanine yields a significant uptake with PPDA, whereas pyrocatechol does give a partial uptake amounting to approximately half that obtained when the alanine side chain is also present. Whether the alanine plays a role in feeding electrons to the reaction sites or merely assists in molecular orientation in the formation of the resulting complex is un-

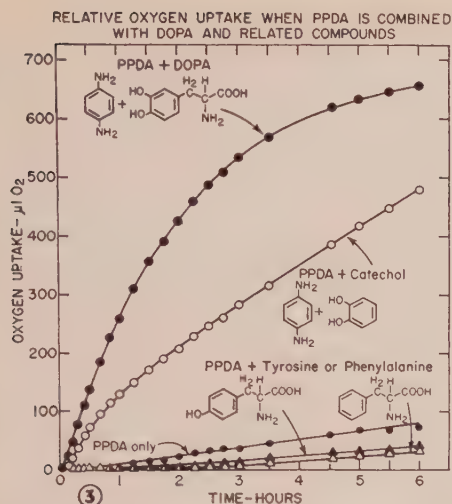


FIG. 3. Relative oxygen uptake when PPDA is combined with DOPA and related compounds. (PPDA, DOPA, catechol, phenylalanine, 0.01 M; 0.05 M phosphate buffer, pH 7.4.)

certain. Although no systematic study has been made of compounds related to DOPA, approximately 150 PPDA-related aromatic amines were examined for specific combination with DOPA from the standpoint of manometric oxygen consumption. Only a few were found active under the experimental conditions employed successfully with PPDA. The

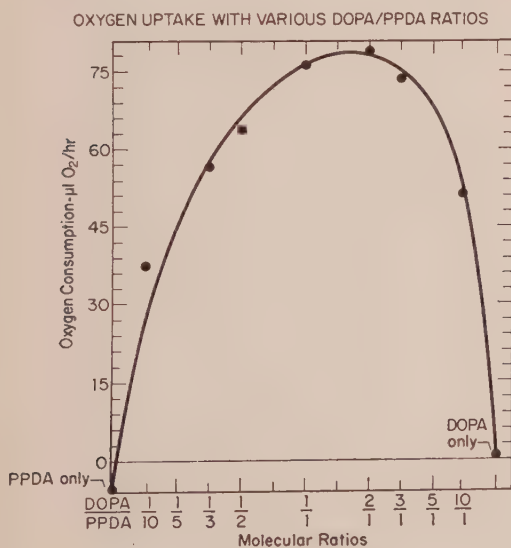


FIG. 4. Oxygen uptake as a function of DOPA-PPDA molecular ratio. (DOPA and PPDA, 0.01 M; phosphate buffer, 0.05 M, pH 7.4.) No NaOH employed for CO_2 absorption.

significantly active compounds were *p,p'*-diaminodiphenylamine, *p,p'*-diaminodiphenylamine-*o*-sulfonic acid, 2,4-toluenediamine, 2,3-diaminotoluene, 2,4-diaminobenzoic acid, and *N,N*-dimethyl-*p*-phenylenediamine.

Fig. 4 illustrates the activity curve obtained by varying the molecular ratio of DOPA to PPDA. While high activity is obtained at ratios of 1:1, 2:1, and 3:1, the peak of activity, at 1 hour, occurs at 2:1, which, together with other data, suggests the formation of a complex, possibly a polymer composed of the basic molecules of DOPA and PPDA. The detailed chemistry of this reaction is still under investigation. Fig. 5 shows

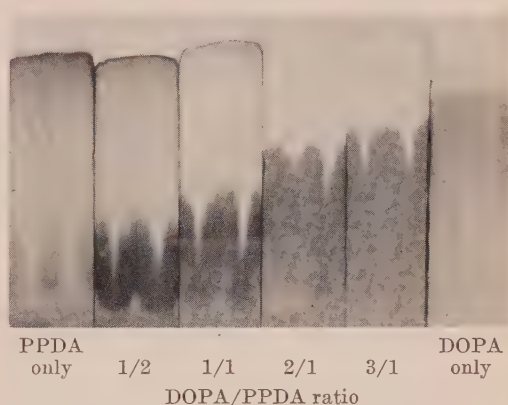


FIG. 5. Individual chromatograms of the pigment produced by various DOPA-PPDA ratios. (PPDA and DOPA, 0.01 M; phosphate buffer, 0.05 M, pH 7.0.)

the relative amounts of pigment formed in the above experiment and its differential behavior in a simple chromatographic system employing a type of capillary analysis(7,8). In contrast to the hyperbolic peaking of the oxygen consumption curve, the R_f values of the migrating pigment increase to the right with increasing DOPA as long as catalytic amounts of PPDA are present. Correlated with this, it was noted that the pigment formed with higher ratios of PPDA precipitated more readily, forming insoluble aggregates in the bottom of the chromatographic beaker.

The detection of an *in vitro* oxygen consuming reaction involving an intracellularly produced metabolite of melanoma tissue sug-

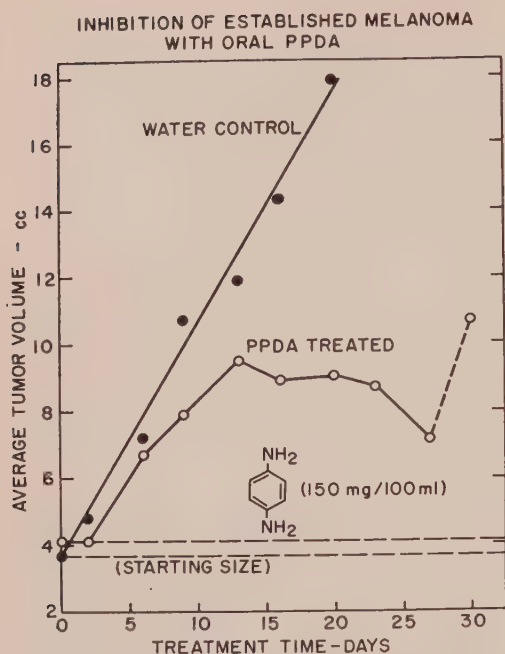


FIG. 6. Inhibition of established mouse melanoma with oral PPDA. (PPDA, 150 mg/100 ml of drinking water; tumor, 27-day post-implant of Cloudman S91 melanoma.)

gested the possibility of interfering selectively with the metabolism of this malignant cell if the reaction could be generated *in vivo*. Fig. 6 shows the inhibition of established Cloudman S91 mouse melanoma when hosts were treated orally with 150 mg of PPDA per 100 ml of drinking water. Treatment was started 27 days after implant of the tumors when the average tumor volume was approximately 4 cc. The tumor sizes illustrated are the means of 10 tumors in each group at the time treatment started, with the number decreasing to 1 or 2 animals at the end of the experiment. The treated animals survived approximately 30% longer than the controls. It should be noted that the ortho and meta isomers gave significantly greater melanoma inhibition. Further details on these compounds will be described separately.

Discussion. Although PPDA and its oxidation products have been implicated in certain combinations with enzymes and other proteins (9,10), data recently reported from this laboratory provide evidence of a special affinity of PPDA for melanoma tissue or its products

(11). Specifically, when melanoma bearing mice are injected with an acutely lethal dose of PPDA they survive significantly longer than do their non-tumor controls. In view of the findings reported here the most consistent explanation of this phenomenon seems to be an *in vivo* detoxification of the injected PPDA by its combination with DOPA-like components present in the melanoma tissue (Fig. 1) or in the peripheral blood (Fig. 2), or by both, since the products of similar combinations incubated *in vitro* are less toxic to the host than are the individual components. The chemotherapy experiments with transplanted pigmented tumors were stimulated by the above data and its implications. However, the verification of a mechanism whereby an intratumoral combination of PPDA with DOPA-like metabolites disrupts the metabolic integrity of the malignant cell and thus causes its inhibition or destruction is a complicated undertaking and necessitates further study. At present the most persuasive argument in its favor is the observation of tumor inhibition by PPDA and related compounds.

Summary. A relatively vigorous oxygen consuming, non-enzymic reaction occurs at physiological temperatures between *p*-phenylenediamine (PPDA) and dihydroxyphenylalanine (DOPA) when the 2 compounds are mixed in aqueous solution at pH 7.0 to 7.5, with maximum activity, based on oxygen consumption, obtained at a DOPA-PPDA molecular ratio of approximately 2:1. The reaction has been studied manometrically with pure compounds and with various melanoma components, such as are contained in mouse melanoma extract and in plasma and urine from patients with disseminated malignant melanoma. The oxidation observed with various melanoma components is thought to be identical or analogous to the PPDA-DOPA reaction found with the pure substances since in all instances the reaction is both KCN and heat insensitive. Preliminary data are given to illustrate the possibilities of exploiting such reactions for diagnostic and chemotherapeutic purposes.

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Production by Various Fats of Myocardial Necroses in Humorally Conditioned Rats.* (23940)

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When rats are simultaneously treated with certain highly active corticoids, such as 2 α -methyl-9 α -chlorocortisol (Me-Cl-COL)[†] plus sodium phosphates (Na₂HPO₄, NaH₂PO₄), they invariably succumb with clinical manifestations of acute cardiac death, and autopsy regularly reveals large, yellowish infarct-like necrotic patches in their myocardium(1). Exposure to stress (e.g., neuromuscular exertion) can precipitate the development of such infarct-like myocardial necroses, in rats conditioned by pretreatment with small doses of corticoids and electrolytes(2). These cardiac lesions are not accompanied by any morphologically demonstrable evidence of coronary artery occlusion, yet they resemble cardiac infarcts in some respects. Since, during the last few years, a great deal of evidence has accumulated in support of the concept that certain fats may precipitate the development of true cardiac infarcts in man(3), we wanted to verify whether the production of myocardial necroses by corticoids plus sodium phosphate in the rat could likewise be precipitated by the oral administration of fats.

Materials and methods. One hundred forty female Sprague-Dawley rats, with an average

initial body-weight of 99 g (range: 93-109 g), were subdivided into 14 equal groups as indicated in Table I. Me-Cl-COL was injected subcutaneously, in the form of its acetate, as a micro-crystal suspension of 100 μ g in 0.2 ml of water, once daily. Na₂HPO₄ was administered by stomach tube, at the dose of 1 mM (141.98 mg) in 5 ml of water, twice daily. The various fats were given twice daily, by stomach tube, at the doses indicated in Table I. The experiment was terminated after 6 days, by killing the survivors with chloroform. The hearts of all animals were fixed in Susa solution, for subsequent staining with hematoxylin-eosin. Since nephrocalcinosis frequently accompanies the myocardial necroses produced by Me-Cl-COL plus Na₂HPO₄, specimens of all kidneys were fixed in neutral formalin for the subsequent histochemical demonstration of calcium deposits by the v. Kossa's silver nitrate technic. Both organs were embedded in paraffin.

Results. The principal results of these experiments are summarized in Table I, which lists the mean final body-weight, the severity (graded in terms of an arbitrary scale of 0 to 3) of cardiac necroses and nephrocalcinosis, as well as the percentual incidence of these changes, and the mortality rates.

Treatment with as much as 1 ml of corn oil, twice daily, alone (Group 1) or in com-

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[†] Kindly supplied by Upjohn Co.

TABLE I. Effect of Various Fats upon Myocardial Necroses.

Group	Treatment	ml	Final body wt (g)	Cardiac necroses		Nephrocalcinosis		Mortal- ity (%)
				Incidence (%)	Grade (scale 0-3)	Incidence (%)	Grade (scale 0-3)	
1	Corn oil	1.0	94.5 ± 2.4	0	0	0	0	0
2	Me-Cl-COL + Corn oil	1.0	84 ± 2.1	0	0	0	0	0
3	Me-Cl-COL + Na ₂ HPO ₄	5.0	82 ± 2.5	20	.5 ± .32	50	.6 ± .23	20
4	<i>Idem</i> + corn oil	.5	81 ± 2.3	80	1.7 ± .32	70	1.5 ± .40	30
5	" + "	1.0	82 ± 2.7	100	2.5 ± .25	89	1.7 ± .34	100
6	" + peanut oil	.5	83 ± 2.8	50	1.1 ± .44	60	.8 ± .28	20
7	" + "	1.0	80 ± 1.9	100	2.6 ± .27	80	1.0 ± .2	80
8	" + olive oil	.5	85 ± 2.1	60	1.6 ± .48	70	1.3 ± .37	40
9	" + "	1.0	84 ± 1.9	90	2.7 ± .30	100	1.9 ± .23	90
10	" + pork fat	.5	84 ± 2.8	60	1.6 ± .48	80	1.4 ± .34	40
11	" + "	1.0	80 ± 2.5	89	2.4 ± .38	89	2.1 ± .34	70
12	" + chicken fat	.5	83 ± 1.6	80	1.6 ± .34	80	1.6 ± .30	60
13	" + "	1.0	82 ± 1.6	100	2.9 ± .1	90	1.4 ± .23	100
14	" + paraffin oil	1.0	81 ± 1.8	10	.1 ± .1	30	.3 ± .15	10

bination with Me-Cl-COL (Group 2), produced no cardiac necroses, nephrocalcinosis or mortality. In these respects, Me-Cl-COL plus Na₂HPO₄, without additional treatment (Group 3), was also only very moderately effective because of the short duration of the experiment. This is in sharp contrast with the intensity of the cardiac necroses and nephrocalcinosis and with the high mortality rate in all the animals which, in addition to the steroid-electrolyte treatment, were also given plant or animal fats (Groups 4-13). On the other hand, the inabsorbable mineral oil did not significantly accentuate the production of organ changes, nor did it increase the mortality in rats concurrently treated with Me-Cl-COL plus Na₂HPO₄ (*cf.* Group 14 with Group 3).

The degree of nephrocalcinosis ran roughly parallel with the severity of the cardiac necroses.

Discussion. It would not be permissible to draw any definite conclusions from this experiment concerning the probable effect of various fats upon the incidence of true cardiac infarcts such as occur in man. It is clear, however, that under our experimental conditions,

both plant and animal fats precipitated the production of myocardial necroses by short-term treatment with Me-Cl-COL plus Na₂HPO₄. This sensitization could not have been due to the stressor effect of daily gavages with oily material, since similar treatment with paraffin oil proved to be ineffective. Also, it is significant that the animals of all groups lost some body-weight during the experiment; hence, the fats could not have predisposed the cardiac muscle to the production of necroses, by placing, upon the heart, an excess burden due to an increase in body mass.

Summary. Experiments on rats indicate that the degree of myocardial necroses and nephrocalcinosis normally produced by combined treatment with 2 α -methyl-9 α -chlorocortisol (Me-Cl-COL) and Na₂HPO₄ is considerably increased by oral administration of plant or animal fats, but remains uninfluenced by mineral oil.

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Effect of Alcohols on Lecithinase of *Clostridium perfringens*.^{*} (23941)

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Kushner has reported recently(1) that the presence of certain alcohols in the growth medium inhibited markedly the formation of lecithinase by cells of *Bacillus cereus* while having little effect on the growth of the organisms. The data reported revealed that the effect of the alcohols was on the synthesis of lecithinase rather than on the preformed enzyme.

Inasmuch as we have been concerned with a study of the influence of nutrition on synthesis of lecithinase by *Clostridium perfringens*(2), it appeared desirable to extend the experiments of Kushner to include this organism.

Materials and methods. The organism studied was *Cl. perfringens* strain 111-3 and was maintained by transfer every 3-4 weeks in nutrient broth containing chopped beef heart. The experimental medium contained 2 g of proteose peptone No. 3 (Difco), 1 g of sucrose, 0.83 g of K_2HPO_4 , 0.16 g of KH_2PO_4 , 20 mg of $MgSO_4 \cdot 7H_2O$, and 1 mg each of $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$ and NaCl per 100 ml of deionized water and was dispensed in 10 ml amounts into 15 x 125 mm screw cap tubes. The pH prior to sterilization in the autoclave (121°C, 15 min.) was 7.2-7.4. The alcohols were added aseptically to the media at the time of inoculation.

The inoculum was prepared from a 16-20 hr culture grown in the experimental medium at 39°C in an atmosphere of H_2 . All media were boiled routinely in a water bath for 10 min. and cooled quickly under running water prior to inoculation in order to expel dissolved O_2 . The inoculum culture was centrifuged, the bacterial cells washed once with sterile medium, resuspended in 10 ml of the medium and 2 drops of this suspension used to inoculate each experimental tube. Growth was measured turbidimetrically after 4-5 hr incubation in a Coleman Junior Spectrophotometer

at a wavelength of 650 m μ employing a medium blank.

Lecithinase (*alpha* toxin) was measured by the Nagler reaction. Lecithovitellin was prepared by mixing one egg yolk in 500 ml of 0.9% salt solution followed by adsorption with 20 g of Norite. This was accomplished by shaking the flask on a mechanical shaker for 30 min. followed by centrifugation to remove the Norite. The remaining solution was then passed through a Selas filter and stored in the refrigerator. Toxin titers were determined by mixing 3 ml each of lecithovitellin and saline and 2 ml of culture supernate obtained by centrifugation and incubating at 43°C for 1-2 hrs. The culture supernates were adjusted to pH 7 in order to minimize non-specific reactions. The resulting turbidities were measured in the Coleman at 650 m μ . Control tubes were identical except for the addition of polyvalent gas gangrene antitoxin (Lederle) to the culture supernate before it was mixed with the lecithovitellin.

Results. The data for two representative experiments are presented in Table I. Methanol and ethanol were approximately equivalent in activity both with regard to growth and toxin inhibition. At concentrations of 0.5 and 1.0% growth was reduced 10-34% (av 20%) while lecithinase activity was inhibited 35-71% (av 52%). At a concentration of 2% both methanol and ethanol caused an inhibition of lecithinase of 90% or greater with a concomitant decrease in growth approximating 50%. Propanol and butanol were decidedly more toxic to growth and toxin formation than the lower chain alcohols but even with these alcohols the effect on lecithinase activity was approximately twice the effect on growth especially at the lower concentrations.

The results cited do not permit a distinction between possible effects of the several alcohols on the synthesis of lecithinase or the stability of this toxin. It was therefore desirable to study the action of the alcohols on preformed

^{*} Supported in part by a contract between the Office of Naval Research, Dept. of the Navy, and the Univ. of Minnesota.

toxin. This was accomplished by growing the organism in media free of alcohol, recovering the supernatant culture fluid after removal of the cells by centrifugation and then adding the alcohols individually to aliquots of the culture supernate. The results of such studies (Table II) revealed that the alcohols had no effect on stability of preformed toxin hence suggesting that their effect during growth is

TABLE I. Effect of Alcohols on Growth and Lecithinase Production of *Clostridium perfringens*.

Additions, %	Growth, O.D.*	Toxin, O.D.†	Growth inhibition, %	Toxin inhibition, %
none	.68	.45		
methanol, .5	.58	.24	14.7	46.6
1.0	.50	.14	26.5	68.9
2.0	.35	.03	48.5	93.4
ethanol, .5	.56	.23	17.6	48.8
1.0	.45	.13	33.8	71.1
2.0	.35	.01	48.5	97.8
n-butanol, .5	.40	.03	41.2	93.4
1.0	.05	.00	92.6	100.0
none	.57	.57		
methanol, .5	.51	.37	10.5	35.1
1.0	.43	.22	24.5	61.4
2.0	.25	.06	56.1	89.5
ethanol, .5	.50	.37	12.3	35.1
1.0	.43	.31	24.5	45.6
2.0	.22	.05	61.4	91.2
iso-propanol, .5	.43	.28	24.5	50.9
1.0	.30	.12	47.4	78.9
2.0	.03	.00	94.7	100.0
n-butanol, .5	.25	.03	56.1	94.7
1.0	.17	.00	70.2	100.0

* Optical density at 650 m μ after 5 hr incubation at 39°C in an atmosphere of H₂.

† Optical density at 650 m μ of culture supernate after mixing with lecithovitellin and incubating at 43°C for 90 min.

Other details as described in text.

TABLE II. Effect of Alcohols on Preformed Lecithinase of *Clostridium perfringens*.

Additions	Lecithinase activity	
	Exp. 1 O.D.*	Exp. 2 O.D.
none	.48	.29
methanol	.50	.28
ethanol	.43	.27
iso-propanol	.48	.26
n-butanol	.50	.29

* Optical density at 650 m μ of culture supernate after mixing with lecithovitellin and incubating at 43°C for 2 hr. Alcohols added to culture supernate in final conc. of 2%. Other details as described in text.

exercised by inhibiting toxin synthesis.

The results with *Cl. perfringens* were roughly parallel to those obtained with *B. cereus*(1) except that the several alcohols were decidedly more toxic for growth of the former organism than the latter. However, it is manifest that the inhibitory effect of the alcohols on toxin synthesis was far greater than the effect on growth suggesting a preferential effect on this function rather than a general metabolic inhibition.

Summary. A study of the effects of several alcohols on growth and lecithinase synthesis by *Cl. perfringens* revealed that although both functions were inhibited by appropriate concentrations, toxin synthesis was considerably more susceptible than growth. In general the inhibitory effects of the alcohols increased with increasing molecular weight.

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Quantitative Changes in Lipid Composition of Tissues Infected with Fowlpox Virus.*† (23942)

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Numerous investigations have been made of the effects of animal viruses on metabolism of their hosts. Bauer(1) and Putnam(2) have reviewed much of the previous work. Many of these studies have been concerned with respiration and with nucleic acid and phosphorus metabolism. No investigations have been concerned primarily with lipid metabolism. It has been demonstrated(3) that lipids in stainable quantities accumulate in the inclusion bodies found in chick skin or chorioallantoic membrane after infection with fowlpox virus. In order to understand better the pathogenesis of this infection a study was made of lipid distribution of normal and infected chick skin and chorioallantoic membrane. The results indicate that quantitative changes in lipid composition of these tissues accompany infection with fowlpox virus.

Materials and methods. *Virus.* A field strain of fowlpox virus obtained from Dr. E. R. Doll, University of Kentucky was employed. In this laboratory the agent had been passed 5 times in chick scalp. To procure a large amount of inoculum, the last passage was made in 4 dozen chicks. This stock material was stored in small amounts at -20°C until used. *Fertile eggs* were obtained from local hatchery. Eleven-day-old eggs were inoculated on the chorioallantoic membrane (CAM) by the classical window method and incubated separately from controls, both groups at 37°C . Control CAM were collected at 16 days. Whole membranes were collected from control eggs, whereas only lesions were collected from infected eggs. Analyses were made upon 5 groups each of normal and in-

fectected CAM. Each group consisted of from 75-85 membranes. *Day-old "Hyline" cockerels* obtained from local commercial hatchery were inoculated by scrubbing 1:5 or 1:10 saline dilution of the virus into the plucked scalp. Normal and infected tissues were collected from chicks at 7 days of age. Normal tissues consisted of scalps of 4 groups of birds (50-60/group), while infected scalps were obtained from 5 groups of infected chicks (50-60 group). Chicks were fed a balanced "starter" ration and water *ad libitum*. *Extraction.* Specimens of CAM and skin were frozen within 30-60 minutes after collection and maintained at -45°C . Subsequently they were dried to constant weight from frozen state by lyophilization. Extraction of lipid was performed by boiling the tissues 30 minutes in 95% ethyl alcohol followed by boiling for 30 minutes in Bloor's solvent and consequent continuous extraction for 48 hours by diethyl ether in a Soxhlet apparatus. Combined alcohol and ether extracts were evaporated to dryness under nitrogen, and lipids extracted exhaustively from the residue with petroleum ether (B.P. $30-60^{\circ}\text{C}$). After evaporation of petroleum ether under nitrogen, total lipids were dried to constant weight. The weighed lipids were redissolved in known volume of petroleum ether, usually 50 to 100 ml, and aliquots taken for various determinations. All *chemical determinations* were done in triplicate. *Cholesterol.* The Sperry-Webb(4) modification of Schoenheimer-Sperry method for cholesterol was used for determinations of free and total cholesterol. Esterified cholesterol was calculated as the difference between free and total cholesterol. The amount of fatty acid associated with cholesterol was estimated using the formula of Bloor(5). Color densities were read in Bausch and Lomb colorimeter at $625\text{ m}\mu$ and compared with similarly treated samples of known cholesterol concentration. *Free fatty acids.* Suitable

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† Presented in part at annual meeting of Soc. Am. Bact., Detroit 1957. Taken in part from Ph.D. research of W.M.T. at Vanderbilt Univ. School of Medicine.

TABLE I. Percentage Composition of Chick Skin Lipids.

Constituents	Normal skin	Infected skin	P*
Total lipid†	23.95 ± 1.24‡	19.46 ± .68	.02
Total cholesterol	9.95 ± 1.02	13.46 ± .75	.05
Free "	6.65 ± .72	7.82 ± .29	.10
Esterified "	3.28 ± .29	6.49 ± .40	.001
Phospholipid	18.48 ± 2.21	23.18 ± 1.54	.10
Free fatty acids	12.38 ± .40	15.78 ± .98	.025
Total " "	73.50 ± 1.19	60.84 ± 3.34	.02

* Student's t-test for significance. If P is less than .05, the change is considered significant.

† Calculated on basis of dry CAM wt.

‡ Mean ± stand. error.

aliquots of petroleum ether extracts were evaporated to dryness, dissolved in ethanol and titrated with 0.02 N NaOH. The factor used for converting titration results (milliequivalents) to mg was 0.282, the theoretical value for oleic acid. *Total fatty acids* were titrated as described above after saponification and extraction by modification of the method of Kelsey (6). *Concentration of phospholipid* was estimated by determining lipid phosphorus by the method of Fiske and Subbarow (7) and multiplying the value in mg by 25. The amount of fatty acids found in phospholipids was calculated from the phospholipid value using the formula suggested by Bloor (5). A check of the extraction procedure was performed by dissolving a weighed sample of tripalmitin in petroleum ether and subjecting it to extraction procedures previously described for the tissue samples. Titration of free fatty acids determined that there was no increase in fatty acids due to extraction procedure. To test for presence and activity of lipase in tissues, individual tissues from one group each of normal CAM and chick scalps, respectively, were frozen immediately after collection in containers immersed in dry ice and acetone. Two similar groups of control tissues were stood at room temperature for one hour after collection and then frozen. Both groups of tissues were carried through same procedure of lyophilization and extraction. Titration revealed that the content of free fatty acids was the same, within experimental error.

Results. Percentage composition of lipid extracted from normal and infected chick skin is given in Table I. Fractions undergoing significant changes after infection include to-

tal cholesterol, esterified cholesterol, and free fatty acids, all of which increased, and total fatty acids, which decreased. When results are calculated on a dry skin weight basis total lipid, esterified cholesterol and total fatty acid values show significant change. The range in concentration of other components reveals extent of physiologic variation.

Percentage composition of normal and infected CAM lipid is indicated in Table II. Concentrations of free cholesterol and phospholipid show significant decreases in infected membranes in contrast to increased values for esterified cholesterol. When results are calculated on basis of dry weight all components show significant change except phospholipid.

Discussion. Addition of individual lipid fractions reveals that 105% of the lipid in chick scalp, and 101% of that found in chorioallantoic membranes was recovered. These figures are within experimental error of the methods.

Statistical analysis of the data suggests that there is an alteration in lipid metabolism of tissues after infection with fowlpox virus, as indicated by quantitative changes in tissue lipids. That this effect may not be specific for fowlpox is suggested by the work of Cohn (8) who found decreased amounts of phospholipid phosphorus in CAM infected with influenza virus. Cornatzer (9) found a decrease in total lipid in papillomata produced in rabbit skin by the Shope virus, but phospholipid phosphorus increased in the lesion. Miroff, Cornatzer and Fischer (10) found while studying phosphorus metabolism of poliomyelitis-infected HeLa cells that although there was no net increase in phospholipid, there was an increased uptake of P³² into the phospholipid

TABLE II. Percentage Composition of Chorioallantoic Lipids.

Constituent	Normal CAM	Infected CAM	P*
Total lipid†	15.98 ± .74‡	23.72 ± .56	.001
Total cholesterol	17.60 ± .93	16.22 ± .50	.20
Free "	16.90 ± .86	4.30 ± .14	.001
Esterified "	.71 ± .09	11.85 ± .44	"
Phospholipid	53.36 ± 1.38	32.04 ± 1.50	"
Free fatty acids	5.38 ± .70	7.18 ± .29	.10
Total " "	47.66 ± 4.00	54.14 ± 3.72	.25

* Student's t-test for significance. If P is less than .05, the change is considered significant.

† Calculated on basis of dry CAM wt.

‡ Mean ± stand. error.

of cells. Other work of a similar nature has been summarized by Blank(11).

At present the site of metabolic alteration is not known. It is possible that infected cells are capable of metabolizing lipids in an abnormal fashion under the influence of the virus. An alternate possibility is that the liver of chick and embryo may have been metabolically damaged by the virus. Goodpasture(3) pointed out that there is a viremia in fowls infected with fowlpox, despite the fact that epithelium and mucous membranes are the only tissues which show lesions. Cohn(8) employed de-embryonated eggs in his study of phosphorus metabolism as affected by influenza virus infection and noted a decrease in CAM phospholipid phosphorus, the same effect noted in our study in which embryonated eggs were used. Gould and Taylor(12) have shown that the skin of certain species can synthesize cholesterol at a rate comparable to that in liver. Similar information concerning the CAM is not available.

In CAM, the virus seems to have produced a generalized increase in lipid with the exception of phospholipid and free cholesterol fractions. This increase may reflect an increased synthesis of lipid or a decreased ability of tissue to utilize this class of compounds. Changes in phospholipid and cholesterol may be explained by postulating that free cholesterol and the fatty acids formerly esterified to phospholipids have been used to form cholesterol esters. Apparently there has been an increased accumulation of fatty acids, some of which are present as free fatty acids.

Summary. 1) The normal lipid composition of chick skin and of chorioallantoic membrane is reported. Evidence is presented which indicates that there is an alteration in lipid metabolism of these tissues when infected with fowlpox virus. 2) This evidence

consists of: (1) On dry weight basis, infection of the chick resulted in decreased amount of total skin lipids, the greater part referable to total fatty acid fraction. The level of esterified cholesterol became elevated. (2) Partition studies of chick skin lipid revealed that concentration of total cholesterol, esterified cholesterol and free fatty acids increased in contrast to lowered total fatty acid values. (3) Dry weight analyses of infected chorioallantoic membrane demonstrated a general increase in lipid components with the exception of free cholesterol and phospholipid, which decreased and were unchanged, respectively. (4) Partition of CAM lipid disclosed that free cholesterol and phospholipid decreased in percentage while esterified cholesterol exhibited a 15-fold increase during infection.

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Plaque Assay of Measles Virus on *Erythrocebus patas* Monkey Kidney Monolayers.* (23943)

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Since the first isolation of measles virus in human and rhesus kidney cells by Enders and Peebles(1), the virus has been grown in human amnion and cancer cells and in chick embryos(2-6). During our investigation of the viral susceptibility of kidney cells from the African red grass monkey (*Erythrocebus patas*)(7,8), it was found that measles virus could grow readily in patas cultures and produce distinct plaques with clear centers and sharp boundaries.

Materials and methods. Measles virus† used was the Edmonston strain which had been through 23 passages in human kidney culture(1), 1 passage in HeLa culture, and 7 passages in Hep-2 cells(4). Measles anti-serum: pre- and post-inoculation sera were obtained from monkeys immunized with a single injection of measles virus grown in Hep-2 cell culture. Paired sera from measles patients were obtained from Greenland.‡ Bottle cultures of monkey kidney monolayers were prepared and overlaid as described previously (7,9). The neutralization test was performed by mixing equal amounts of virus containing approximately 50-100 plaque forming units (PFU) with 0.5 ml of serum at different dilu-

tions. The mixture was incubated at room temperature for one hour, and 0.2 ml of the mixture was inoculated per bottle.

Results. Fig. 1 and 2 show the plaques produced by measles virus at 3 tenfold dilutions in patas bottle cultures, 6 and 10 days after seeding. Small but distinct plaques were obtained as early as 5-6 days after inoculation. After the size of a plaque increased to 2-3 mm in diameter it became very distinct. Titration of measles virus in patas cells by the plaque method and also by CPE (cytopathogenic effect) in tubes is shown in Table I.

Measles plaques were also obtained in rhesus bottle cultures but only if the cell sheets were in excellent condition. Even so, the plaques were not as clear, and the boundaries were not as distinct as those shown on patas monolayers (Fig. 3). In simultaneous tests of cells grown out under identical conditions, plaque counts in rhesus cultures were about 10% of those in patas cultures. Quantitative aspects of the propagation of measles virus in a variety of cell lines including patas cells will be reported by Black. A study of the sequence of cytological changes correlated with

TABLE I. Titration of Measles Virus by Plaque Formation and by Cytopathogenic Effect (CPE).

Days after inoc.	PFU/0.1 ml inoculum					CPE*				
	10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	1	0	0	0	0
3	0	0	0	0	0	4	1	0	0	0
6	> 100	59	7	0	0	4	4	2	0	0
7	> 1000	133	21	2	0					
10	c	s-c	26	5	0	4	4	4	1	0
14						4	4	4	4	2

* No. of tube cultures showing CPE. Four cultures inoculated at each dilution.

c = confluent; s-c = semiconfluent.

* Aided by grant from Nat. Fn. for Infantile Paralysis.

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† Measles virus and immune serum kindly supplied by Dr. Francis L. Black.

‡ Obtained through courtesy of Dr. Biggo E. Bech of the Serum Inst., Copenhagen, Denmark.

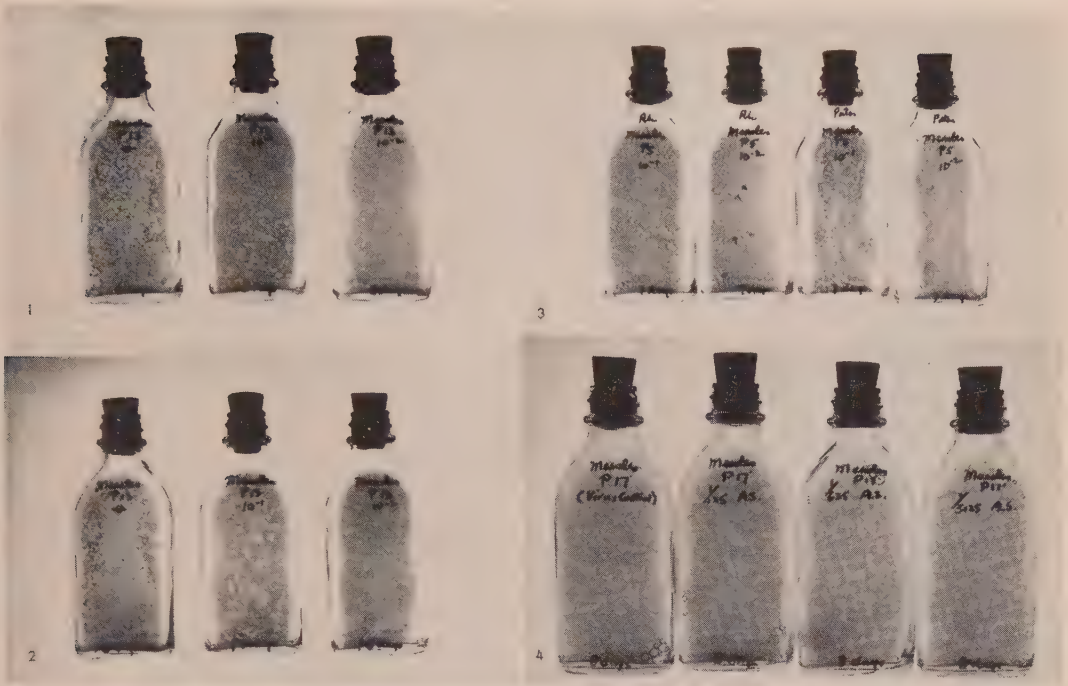


FIG. 1. Quantitative titration of measles virus (13th passage in patas cultures) 6 days after seeding serial 10-fold dilutions, .1 ml/3-oz bottle.

FIG. 2. As Fig. 1 but 10 days after seeding.

FIG. 3. Plaque formation of measles virus in rhesus cultures (2 bottles at left) and patas cultures (2 bottles at right), 7 days after seeding.

FIG. 4. Neutralization test by plaque method. Bottle on left contains challenge dose of measles virus (17th patas passage). Next 3 bottles contain 0.2 ml of virus-serum mixture, at serum dilutions of 125, 625, and 3125, respectively. The virus was neutralized completely at the 2 lower dilutions, and there was 90% plaque reduction at the highest dilution.

the growth cycle of measles virus in patas cells will be published separately, together with Ruth Kleinfeld.

Neutralization of measles virus (both the 4th and 17th passage of measles virus in patas cells) by specific measles monkey antiserum indicated that the patas-passaged measles virus and the Hep-2 passaged measles virus are still antigenically the same. The neutralization end point was obtained on the 7th day of incubation. After 4 passages in patas cells, the virus was neutralized by antisera obtained from 2 immunized monkeys (Table II). The

titer of antiserum obtained from monkey 8628 was 1:400 when tested by the conventional tube method, using Hep-2 cells; however, 80% plaque reduction was obtained at a serum dilution of 1:3125. Fig. 4 illustrates another test by the plaque reduction method, with the same serum versus the 17th patas-passaged virus.

Neutralization of the patas-passaged measles virus was also tested with human serum samples. Table III gives a typical result of a measles neutralization test. The sera were heated at 56°C for 30 minutes before use. No neutralizing antibody was observed in the serum sample taken prior to symptoms, but neutralization occurred at 1:25 of the second serum sample, obtained from the patient one day after the rash appeared. Five days later the serum titer increased to 1:625, after which it fell slightly during the next month.

Discussion. Although cytopathic changes

TABLE II. Neutralization of Measles Virus in Its 4th Patas Passage by the Plaque Method.

Measles immune monkey No.	Control, PFU per bottle	PFU in presence of serum dilutions:				
		1:5	1:25	1:125	1:625	1:3125
8628	57	0	0	0	0	11
9526	57	0	0	0	41	49

TABLE III. Plaque Neutralization Test of Measles Virus by Serum Obtained in Course of Measles Infection.

Date of serum	Phase of disease	No. plaques expected in absence of serum	PFU/.1 ml in presence of serum						
			Undil.	1:5	1:25	1:125	1:625	1:3125	1:15625
9/30	Prior to clinical symptoms	43	20	46	40				
10/ 3	One day after rash	43	0	1	5	24	29	52	
10/ 8	Convalescence	43				0	10	24	48
11/10	"	43		0	0	0	32		

All serum samples were heated at 56°C for 30 min.

of measles (multinucleate giant cells with intranuclear inclusions) were observed in patas cultures 2 days after infection with large doses of virus, with small doses 2-3 weeks were necessary before the final reading could be made. With the plaque method, final results were obtained at the end of one week after inoculation and, in addition, there was no need for changing the medium, as was necessary for the tube method. Quantitative results were easily achieved by counting the number of the distinct small plaques.

Foamy agents(10) have been confused with measles virus because of their characteristic cytopathic changes in rhesus monkey kidney cultures in fluid medium. However, measles-like plaques have not been observed with the foamy agents under agar in rhesus cultures, although the cell sheets were destroyed by the agents. Foamy-like virus has not been recovered from the patas cultures which we have used in our studies over the past 3 years(7,8), except recently from 2 patas monkeys which had been kept in the stock colony for 10 months or longer, and the possibility exists that they might have acquired their infections

from other monkeys in the colony.

Summary. Measles virus produces tiny but distinct plaques with sharp boundaries, in bottle cultures prepared from kidneys of African red grass monkeys (*Erythrocebus patas*). As with other viruses, quantitative assays and neutralization tests with measles virus may now be carried out by the plaque technic.

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Extrapituitary Interaction between Pitressin and ACTH.* (23944)

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Pitressin increases the level of ACTH in the blood(1). It has been assumed that this

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effect is mediated solely by the action of vasopressin on the adenohypophysis(2). The following studies were undertaken to determine whether this assumption is correct.

Methods. Adrenal ascorbic acid depletion

TABLE I. ACTH Activity of Pitressin and Purified Natural Arginine Vasopressin.

Exp.	Test substance	Dose	Adrenal ascorbic acid depletion (mg/100 g of adrenal)		Estimate of ACTH activity, mu/unit†
			Individual responses*	Avg	
A	ACTH U.S.P. standard	1.0 mu†	165, 106, 117, 146, 193, 165	149	.10 ± .02§
		.25 "	39, 58, 29, 27, 48	40	
	Pitressin	5.0 units‡	104, 104, 45, 53, 63, 141	85	
		2.5 "	49, 91, 46, 68	64	
B	ACTH U.S.P. standard	.5 mu	63, 37, 80, 53, 92, 87	69	.09 ± .02
		.25 "	-87, -3, 66, -29, -25, 20, 95	5	
	Purified natural arginine vasopressin	5.0 units	55, 67, 29, 31, 35, 188, 18, 75	62	
		2.5 "	27, 14, 24, 58, 7, 38, 0	24	
C	Pitressin	5.0 units	59, 87, 39	62	
		1.0 "	55, 68, 40, 4, -14, 46	33	
		.2 "	-4, 10, 18, 39, -37	5	
		.04 "	20, -3, 4, 28, -19	6	

* Difference in ascorbic acid concentration in left adrenal removed prior to inj. of test substance and concentration in right adrenal removed one hr after inj.

† Milliunits of ACTH.

‡ Units of pressor activity.

§ ± stand. error.

assays for ACTH were performed 24 hours after hypophysectomy in male rats weighing from 110 to 130 g(3). Hormones‡ were injected intravenously over a period of 5 minutes and were dissolved in 1 ml of 0.9% sodium chloride solution brought to pH 2 by addition of concentrated HCl (acid-saline). Adrenal ascorbic acid depletion assays for ACTH were also performed in rats bearing destructive lesions of the median eminence area of the hypothalamus. The lesions were made in male rats weighing 250 to 300 g with a high frequency alternating current delivered by a stereotaxically placed electrode. Immediately after lesioning the rats were placed in metabolic cages and their 24-hour water consumption and urinary output were determined. Only rats with severe diabetes insipidus (urinary volumes greater than 75 ml) were retained for further study. These animals fail to respond to a variety of stimuli with adrenal ascorbic acid depletion(1). Forty-eight hours after lesioning, the diabetes insipidus rats were divided into 2 groups. Animals of one group were anesthetized with ether and the left adrenal removed. The trachea was intubated and intermittent positive pressure

respiration instituted. The animal's neck was then crushed between the jaws of a large surgical clamp which was placed rostral to the tracheotomy. This procedure completely stops circulatory exchange between the head and the trunk. The animal may be considered decapitated. Following decapitation, 1 ml of acid-saline containing either Pitressin or epinephrine was intravenously infused over a 5-minute period. Thirty minutes after completion of infusion the right adrenal was removed. The second group of rats was treated in the same manner except tracheotomy and decapitation were not performed. The decapitated animals exhibited hypotension at the conclusion of the experiment. The persistence of strong nociceptive reflexes in these animals indicated that despite the hypotension circulation was adequate to maintain some degree of spinal cord function.

Results. Pitressin depletes adrenal ascorbic acid in hypophysectomized rats. This effect is observed only with doses of Pitressin which are much larger than those necessary for demonstration of the antidiuretic and the pressor effects of the hormone. The 5 unit dose was found to be fatal in about 10% of the animals injected. One unit of Pitressin appears to be equivalent to 0.1 milliunit (mu) of ACTH with respect to adrenal ascorbic acid depleting activity (Table I, Exp. A and B). Highly purified natural arginine vasopressin obtained

‡ Grateful acknowledgement is made to Dr. Dan McGinty of Parke, Davis and Co. for generous gifts of Pitressin and of epinephrine, and to Dr. Vincent du Vigneaud for supply of highly purified natural arginine vasopressin (AVN-5).

TABLE II. Potentiation of ACTH by Addition of Pitressin.

Exp.	Test substance	Dose		Adrenal ascorbic acid depletion (mg/100 g of adrenal)		Potentiation with 95% fiducial limits
		ACTH, mu†	Pitressin, units‡	Individual responses*	Avg	
D	U.S.P. ACTH standard	2.0		128, 148, 144, 143, 109, 124	133	1.96 (1.49-2.57)
		1.0		70, 72, 88, 38, 95, 94, 123	83	
		.5		12, 37, 22, 30, 12, 38, 79	33	
	<i>Idem</i> + Pitressin	.50	5.0	140, 141, 128, 124, 125, 114, 134	129	
		.25	5.0	124, 63, 167, 118, 100, 102	112	
E	U.S.P. ACTH standard	2.0		146, 143, 166, 165, 108, 164, 137, 118	143	1.55 (1.14-2.11)
		1.0		143, 118, 86, 130, 117, 98, 86	111	
		.5		96, 70, 65, 70, 112, 51, 57	74	
	<i>Idem</i> + Pitressin	1.2	.2	114, 125, 178, 126, 137, 134, 119, 164	137	
		.6	.2	85, 93, 120, 79, 180, 187, 131	125	
		.3	.2	70, 61, 46, 68, 73, 24, 78, 40	58	

* See Table I.

† Milliunits of ACTH.

‡ Units of pressor activity.

from Dr. V. du Vigneaud exhibits ACTH activity similar to that of Pitressin. This suggests that the ACTH-like action of vasopressin is probably not due to the presence of a contaminant.

Pitressin potentiates the adrenal ascorbic acid depleting activity of ACTH. Comparison of the potency of ACTH administered with 5 units of Pitressin to that of ACTH administered alone yields a potency ratio of 1.96 to 1 (Table II, Exp. D). This potency estimate assumes that 5 units of Pitressin contributes the equivalent of 0.5 mu ACTH. Thus the doses of ACTH employed in the calculation of the potency ratio in assay D were 2.0, 1.0 and 0.5 mu for ACTH alone and 1.0 and 0.75 mu for the combination of ACTH and Pitressin. The potentiation is obviously not accounted for by simple addition. The accuracy of the estimate of potentiation is in part dependent on the accuracy with which the

ACTH activity of Pitressin is determined. To obviate this source of error, a potentiating dose of Pitressin was sought which had negligible ACTH activity. Potentiation of adrenal response to ACTH was found with 0.2 unit of Pitressin. At this dose level Pitressin has no significant effect on adrenal ascorbic acid in the hypophysectomized rat (Table I, Exp. C), but potentiates the action of ACTH by a factor of 1.55 (Table II, Exp. E). In Exp. D and E, the potentiation is statistically significant.

Pitressin depletes adrenal ascorbic acid in the decapitated median eminence lesioned rat. Epinephrine is devoid of activity (Table III). The degree of depletion is significantly less than that observed in the nondecapitated median eminence lesion rat. This difference may be due to Pitressin induced ACTH release by the adenohypophysis of the non-decapitated rat or to the shock-like state of the

TABLE III. Action of Pitressin in Median Eminence Lesioned Rat.

Animal preparation	Test substance	Dose	Adrenal ascorbic acid depletion (mg/100 g of adrenal)	
			Individual responses*	Avg $\pm$ S.E.
Median eminence lesion	Pitressin	5 units	149, 193, 158, 202, 150, 167, 183	172 $\pm$ 8
	"	.5 "	123, 131, 183, 135, 148	144 $\pm$ 11
<i>Idem</i> —decapitated	"	5 "	110, 131, 176, 116, 164, 113, 149, 146, 96, 169, 82, 155, 82	130 $\pm$ 9
	"	.5 "	102, 76, 75, 85	85 $\pm$ 6
	Epinephrine	10 μ g	76, -20, 71, -1, -15, 39	25 $\pm$ 17

* See Table I.

decapitated animal. Regardless of the cause of this difference, the extrahypophysial action of Pitressin is demonstrated.

Discussion. Attention has been focused on the adenohypophysis as the site of action of vasopressin in elevating blood ACTH. The observations reported here and those of Hume and Nelson(4) indicate that vasopressin acts to influence blood ACTH by extrapituitary mechanisms. The *in vivo* potentiation of ACTH by Pitressin may be explained in part by inhibition of a degradative enzyme system (5,6). The possibility cannot be ruled out that part of the potentiation is due to blockade by Pitressin of peripheral binding sites for ACTH. In this connection, Richards and Sayers(7) have demonstrated small stores of ACTH in the kidney of the rat and Higginbotham and Dougherty(8) have interpreted the action of ACTH in enhancing toxicity of polymyxin B as due to competition of the 2 substances for peripheral binding sites. Since highly purified natural arginine vasopressin and synthetic vasopressin(4) both activate the adrenal cortex in the absence of the adenohypophysis, it seems unnecessary to ascribe the ACTH-like activity of Pitressin to contamination with ACTH. Two alternate explanations exist. Vasopressin may act directly on the adrenal cortex or vasopressin may release ACTH from nonadenohypophysial storage sites.

McCann(2) has presented evidence to support the thesis that vasopressin is the corticotropin releasing factor (CRF). On the other hand, Sayers(9) takes the position that CRF is not vasopressin but another basic polypeptide which can be extracted from the hypothalamus. The problem has been complicated by the fact that Pitressin and the hypothalamic extract of Sayers have ACTH activity when assayed in 24-hour hypophysectomized rats. These experimental observations are brought into harmony by the following working concept.

ACTH and a number of other basic polypeptides compete for binding sites both within the adenohypophysis and in extrapituitary tissue. Within the adenohypophysis certain basic polypeptides release bound ACTH by displacing it from intracellular binding sites.

One of these polypeptides is the physiological CRF, another is vasopressin. In extrapituitary tissue basic polypeptides exchange with bound ACTH to bring about release of the tropin. CRF and vasopressin appear to have ACTH activity in the hypophysectomized or decapitated rat by virtue of this mechanism. ACTH and other basic polypeptides also compete for sites in a proteolytic system. The possibility exists that ACTH competes with basic polypeptides at receptor sites in the adrenal cortex.

This concept has proved to be of value in the design of experiments concerned with release of ACTH from the adenohypophysis. The general applicability and theoretical soundness of the concept will be determined by accumulation of additional experimental data. Regardless of the outcome, the conclusion is inescapable that vasopressin and hypothalamic extracts influence the level of ACTH in the blood by extrapituitary actions as well as by release of ACTH from the adenohypophysis.

Summary. Pitressin depletes adrenal ascorbic acid and potentiates the action of ACTH in the 24-hour hypophysectomized rat. The observation that Pitressin depletes adrenal ascorbic acid in the rat with a lesion in the median eminence has been confirmed. Only part of this effect can be explained by release of ACTH from the pituitary since Pitressin will also deplete adrenal ascorbic acid in a lesioned rat which has been decapitated.

The authors gratefully acknowledge the technical assistance of Rosalie Burks and Helen G. Hughes. Dr. E. S. Redgate instructed us in the use of the stereotaxic instrument.

Addendum. After the manuscript was submitted for publication, synthetic arginine vasopressin was found to be as potent as Pitressin per unit of pressor activity in depleting adrenal ascorbic acid in 24-hour hypophysectomized rats.

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Relative Lack of Pharmacologic Action of 3-Methoxy Analogue of Norepinephrine.* (23945)

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Investigations within the past year have indicated that a major pathway for metabolism of norepinephrine in the body is by a combination of methylation of the 3-hydroxy position and oxidative deamination. Armstrong, McMillan and Shaw(1) reported that 3-methoxy-4-hydroxy mandelic acid (VMA) is a major urinary metabolite of norepinephrine and epinephrine in man. Subsequently, Axelrod(2) showed that the 3-hydroxy positions of both of these amines can be methylated by animal tissues *in vitro* and *in vivo* to yield the corresponding 3-methoxy analogues. Axelrod *et al.*(3) have presented evidence that methylation *per se* and methylation followed by oxidative deamination are the major means of metabolizing norepinephrine administered to rats. It is still not certain, however, to what extent endogenous norepinephrine is metabolized in the same manner.

Recently, m-O-methylnorepinephrine (normetanephrine), presumably arising near the site of endogenous formation of norepinephrine, was found in adrenal gland and spleen (personal communication), in the brain of rats after administration of iproniazid(4), and in norepinephrine-producing tumors (pheochromocytomas) of man(5). It seemed important to determine whether this new amine would produce central or peripheral effects on administration to lower animals and to human sub-

jects. The results to be reported suggest that O-methylation of norepinephrine *in vivo* results in its inactivation.

Methods. Studies of the neuropharmacological effects of normetanephrine (NMN) were carried out in cats. Clinical studies were conducted in 2 patients after observations in the dog suggested only weak pressor activity.

A. Pharmacological studies in animals. Studies of the effects of NMN on electrical activity of the brain were carried out in anesthetized cats, and in unanesthetized cats with permanently implanted electrodes. The anesthetized preparations (pentobarbital, 30 mg/kg, intraperitoneally) were employed in studies of the effects of intracarotid administration of NMN on the transcallosal response. In these preparations the transcallosal response was recorded from one suprasylvian gyrus following both maximal and submaximal electrical stimulation of a corresponding point on the contralateral hemisphere. NMN was administered *via* a small polyethylene catheter which had been placed in a thyroid branch of the common carotid artery and advanced until the orifice of the catheter was at the junction of the common carotid artery and the branch which had been cannulated. NMN was dissolved in isotonic NaCl solution; each injection was made in a volume of 1 ml. Chronic preparations with permanently implanted electrodes were employed to study the effects of the NMN on spontaneous cortical activity and on a variety of evoked cortical responses. The cortical response to a

* The normetanephrine was supplied as the hydrochloride through the generosity of Dr. Julius Axelrod and Dr. Bernard Witkop.

brief (10 μ sec) light flash was recorded from the lateral gyrus. In other experiments the cortical response to electrical stimulation of lateral geniculate radiation fibers was studied. The effects of NMN on recruiting responses evoked by 6 to 12/sec stimulation of the nucleus reuniens(6) were also examined. The drugs employed in chronic preparations were administered intravenously. The technic employed in implantation of electrodes was that of Sheatz(7). Studies of the circulatory effects of NMN were carried out in a male, 15 kg mongrel dog under pentobarbital anesthesia. A polyethylene catheter was placed in a femoral vein for injections of drugs, and in a femoral artery for continuous recording of arterial pressure *via* a pressure-transducer-polyviso setup. Single injections of norepinephrine (5 μ g) and NMN (1-4 mg) in 10 ml isotonic NaCl solution were given at 5 to 10-minute intervals.

B. Clinical studies. The human subjects (30-year-old white male weighing 70 kg and 46-year-old white female weighing 76 kg) were patients with uncomplicated labile hypertension. All observations were made with the subjects supine and in the fasting state. After a control period of one to 2 hours, alternate intravenous infusion of norepinephrine and placebo (5% dextrose in water) or NMN and placebo were given at 15 to 30-minute intervals with the subject being unaware of the nature of the injected material. Electrocardiograms (Lead II), radial pulse rate and arterial pressure (arm cuff-auscultatory technic) were recorded at frequent intervals during the control and experimental periods. A symptom questionnaire(8) was employed in the subject (30 yr. male) who received the largest dose (5 mg) of NMN in an attempt to detect any alteration of subjective state.

Results. NMN had no consistent effects on any of the forms of central electrical activity which were studied in cats. In the anesthetized preparations, intracarotid injections of up to 2 mg of NMN were without effect on the transcallosal response. The "spindling" which is characteristic of the spontaneous cortical activity during barbiturate anesthesia was unaffected by NMN. The studies carried out in unanesthetized cats

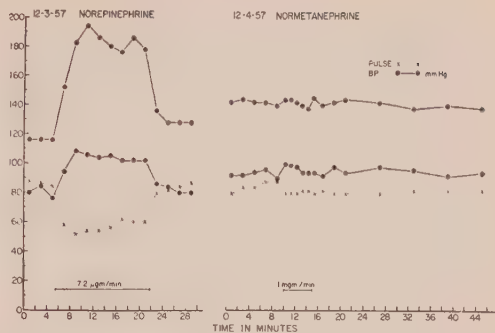


FIG. 1. Effects of norepinephrine and normetanephrine on blood pressure and pulse rate in a human subject. Drugs were administered intrav.

were also negative. NMN in doses up to 10 mg/kg intravenously was without effect on spontaneous cortical activity, recruiting responses, or the primary cortical responses to retinal photic stimulation or electrical stimulation of the lateral geniculate radiations. The gross behavior of the unanesthetized cats was not discernibly altered following intravenous administration of up to 10 mg/kg of NMN.

In the dog, with a maximum dosage of 4 mg NMN (0.25 mg/kg), a slight transient pressor response (+ 25/20 mm Hg) was observed. In contrast, 5 μ g norepinephrine (0.3 μ g/kg) produced a marked pressor response (+ 60/30 mm Hg). This single study suggested that NMN was 1/500th or less as active as norepinephrine on canine arterial pressure.

Varying doses of NMN were administered as 5-minute infusions in the 2 patients, beginning with 25 μ g and to a maximum dose of 5 mg. No physiologic or psychologic reactions were observed. The marked cardiovascular reaction to norepinephrine (36 μ g in 5 minutes) and the lack of any response to normetanephrine (5 mg in 5 minutes) is illustrated in Fig. 1.

Conclusions. Normetanephrine is inert as regards those aspects of central nervous system activity which have been studied in cats, and is a relatively weak pressor agent in the dog. An intravenous dose of 5 mg was inactive with regard to circulatory and psychological effects in man. Although a more detailed study of this compound should be done, the findings presented strongly suggest that O-

methylation at the 3 position is an effective means of inactivating norepinephrine. It will be of interest to investigate the enzymatic mechanisms involved in this unusual methylation process and to attempt to alter it *in vitro* and *in vivo*.

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A New Chemical Method for Measuring Cell Populations in Tissue Cultures.* (23946)

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Among more popular criteria for measuring cell populations in tissue cultures are cell counts, respiration rate, "hematocrit," isotope incorporation, nutrient uptake, desoxyribonucleic acid (DNA) content, and protein content (1-7,9-13). In this report we wish to present a new chemical method for measuring size of tissue cultures, based upon determination of "total purines and pyrimidines" (TPP). TPP is determined simply by extracting washed cultures with hot acid salt solution, and measuring the absorbance of the extract at 268 m μ . While TPP values correlate well with cell counts, mainly we have used DNA as a "standard of reference" for measuring populations. The case for DNA as a measure of cell numbers was summarized by Healy, Fisher, and Parker(6). To evaluate TPP as a measure of cell populations we studied young, old, starved and well-fed cultures, and determined the TPP, DNA and protein in each. As would be expected, TPP and protein both varied somewhat and similarly with respect to DNA. The data indicate that TPP is at least as good as protein for quantitation of cell populations. The virtues of the TPP method are simplicity, reproducibility, stability of absorbance, and a wide range of op-

eration. Ribonucleic acid (RNA) determination is a logical extension of the TPP method, which we hope to employ. If the cells are first extracted with cold trichloroacetic or perchloric acid, the TPP of the residue will consist of only RNA and DNA. RNA then will be equal to the difference between TPP and DNA, both being determined in the same alkaline extract as described below.

Methods. Cell cultures. HeLa S-3, was obtained from Tuskegee Institute. The McCoy strain of human cells was kindly supplied by Prof. C. M. Pomerat. Strain L, NCTC929-209-7 was obtained through the kindness of Dr. Wilton R. Earle, Nat. Cancer Inst. Stock cultures were fed and divided twice weekly. Human cells were cultured in a synthetic medium very similar to Eagle's, with human serum non-dialyzable fraction equivalent to 10% serum, supplemented with 1 g of Difco Bacto Tryptose/liter. Strain L was cultured in the same synthetic medium with 10% horse serum instead of human serum. Incubation was at 37°C. Replicate cultures were planted by Earle's apparatus (4). Strain L and McCoy were suspended by mechanical agitation; HeLa required trypsin treatment. Cultures were planted in 18 x 150 mm test tubes graduated for 5 ml volume, with a conically tapered bottom and an indentation 7 mm deep and 15 mm from mouth

*We are grateful to Henry Kokko for technical assistance and to Jack Arzoomanian for the drawings.

of tube.[†] During incubation the tubes were horizontal. They were placed in special racks with the indentation over a rail which prevented rolling. Also, the indentation kept the media away from the silicone rubber stoppers. Each culture contained 2.5 ml of medium. For the chemical determinations, cultures must be rinsed carefully with physiological salt solution. In our experience equally good results may be obtained with 0.9% sodium chloride, adjusted to pH 4.5 with phosphoric acid, and Earle's salt solution, pH 7, without sodium bicarbonate. For cultures of 1 to 5×10^6 cells two 5 to 10 ml rinses should be sufficient. Centrifuge at 2000 rpm.

TPP procedure. To each washed culture add 5 ml of 0.1 N sulfuric acid containing 10% sodium chloride. Cover tube with glass cap. Arrange tube almost horizontally so that acid salt solution covers the cells. Heat in live steam 1 hr, cool, adjust to 5 ml volume, centrifuge, and read absorbance of the solution at 268 $m\mu$. From the equation given for DNA by McIntire and Sproull(8) calculate TPP as DNA equivalents. Recently we have modified the procedure as follows: In each washed culture remove the cells from the glass with 2.5 ml of 0.1 N sodium hydroxide. Add 2.5 ml of 0.3 N sulfuric acid containing 20% sodium chloride, mix well, cover with a glass cap, heat in boiling water 15 minutes, cool, mix well, centrifuge and read the absorbance. A volume adjustment will not be necessary if the boiling water is slightly below the level of solution inside the tubes.

TPP, DNA and protein in the same culture.
Reagents and apparatus—0.3 N sulfuric acid containing 20% sodium chloride, 0.4 N sodium hydroxide and other reagents and apparatus required for DNA by the method of McIntire and Sproull(8) and for protein by the method of Oyama and Eagle(10).
Procedure. Follow directions for determination of DNA through the heating in 0.4 N sodium hydroxide and diluting to 0.04 N. Take aliquots and proceed with the DNA determination. For TPP determination take a 0.2 ml aliquot of the .04 N sodium hydroxide extract,

add 0.2 ml of 0.3 N sulfuric acid with 20% sodium chloride. Mix gently, heat in boiling water 15 to 30 minutes, cool, adjust to 0.4 ml volume,[‡] and centrifuge. Read the absorbance of the acid salt solution in a quartz micro cell at 268 and 330 $m\mu$. If the samples are allowed to stand overnight before concentration, the 330 $m\mu$ reading (haze correction) is usually negligible. For protein determination follow the directions of Oyama and Eagle for preparing Lowry's solution C and for standardizing the Folin-Ciocalteu (F-C) reagent. Take an aliquot of .04 N sodium hydroxide culture extract, or protein standard prepared just as the cell extract, and add water to make 1 ml. Mix with 5 ml of solution C and 0.5 ml of F-C reagent and follow through according to Oyama and Eagle. Two protein standards were employed. A purified protein from HeLa cells was the primary standard, human serum albumin was a secondary standard. Results are given in terms of HeLa protein nitrogen(PN).

Results. Significance of TPP determination. Fig. 1 shows absorption curves for TPP solutions prepared by hot acid saline extraction of HeLa and Strain L cells. Solid lines show absorption in the acid salt solution; broken lines show absorption after the acid solutions had been made approximately .05 N to sodium hydroxide. These curves are very similar to the curves for purified DNA treated similarly(8). By the same arguments advanced for the specificity of the DNA method, there is no detectable protein interference, and the TPP value appears to be a measure of purines and pyrimidines and very little else. This value of course includes DNA, RNA, building blocks for these molecules, and co-enzymes. DNA and RNA make up approximately 75 to 80% of the total.

Figure 2 shows that the TPP value is a linear function of cell number when samples of different size are taken from a homogeneous population. For these data aliquots of different size were taken from a stirred suspen-

[†] This culture tube and a smaller one of the same type may be obtained from Bellco Glass Inc.

[‡] These manipulations were done in 8 x 70 mm test tubes calibrated to 0.4 ml by Paul O. Moeller, Universal Instrument Corp., Chicago. For measuring the 0.2 ml aliquots we used micropipets made by Micro-Chemical Specialties Co.

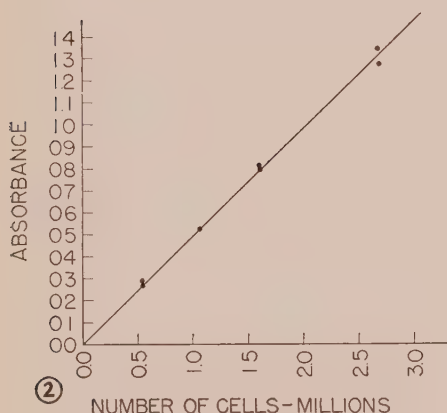
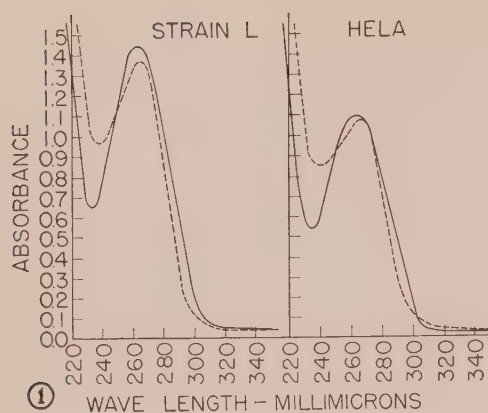


FIG. 1. Absorption spectra of TPP extracts of HeLa and Strain L.

FIG. 2. Relationship of cell number to absorbance of TPP extract of Strain L.

sion of Strain L cells. The cells were centrifuged, washed, and extracted with hot acid salt solution. Each point represents an individual determination, *not an average*.

Relationship of TPP, and PN to DNA in cultures. If TPP and PN are to be used as criteria for measuring tissue cell populations, one must know how constant are these constituents per cell under different conditions of growth. To investigate this question we chose to use the DNA content rather than cell count as a standard of reference, because the constancy of the average DNA per cell in a large population is widely accepted. This means that we will be talking about TPP/DNA and PN/DNA ratios, rather than TPP and PN per cell. To bring out the widest range of ratios that might be encountered in growing cells we analyzed young cultures where the

cells were growing rapidly, older cultures that were crowded and in spent medium, and older cultures that had been fed 24 hours before termination.

The data of Table I show that both TPP/DNA and PN/DNA vary with the culture conditions. A 2-way analysis of variance indicates that the *differences among the 3 groups of cultures of each cell strain* are highly significant. Variance among the 3 groups of each strain was analyzed against variance among replicate cultures within each group. The groups were significantly different at the 0.995 level while there was no significant difference among replicates at the 0.95 level. PN and TPP varied similarly with respect to DNA, but the variations are not great in view of the differences in conditions, and are much less than might be predicted from the work of Davidson and Leslie(3). As would be expected, both PN and TPP were low in the older cultures in which the medium had not been renewed. For many experiments with these cell strains, either TPP or PN would be a suitable measure of cell population, and TPP is at least as good a criterion as PN.

The TPP value may be expressed in several ways. For routine comparisons absorbance units per culture, or per ml of a standard volume of extract, is satisfactory. It may also be expressed in terms of equivalents of DNA, RNA or any purine or pyrimidine which has an absorption maximum near 268 mμ. We chose to use DNA equivalents, because we had on hand a purified preparation of calf thymus DNA.

The center 3 columns of Table I show the DNA, TPP, and PN group average values and the standard deviation of the assays within each group of cultures. In general the standard deviation for PN is greater than for TPP and DNA. The significance of this was not tested statistically.

As a laboratory method TPP has the following advantages over protein determination: 1. TPP absorbance is very stable; it is potentially less capricious than the transitory color of the protein method which requires rigid control of the rate of mixing the reagents. 2. In the TPP method there is no variable blank to be subtracted and no stand-

TABLE I. TPP, DNA and PN in Cultures of Tissue Cells.

Culture, age (days)	No. of cultures	TPP,* μg/culture		DNA, μg/culture		PN,† μg/culture		TPP/DNA μ ± S.E.‖		PN/DNA μ ± S.E.	
Strain L		μ†	S.D.§	μ	S.D.	μ	S.D.				
3	6	44.2	.88	16.5	1.16	45.2	3.18	2.69	.087	2.75	.127
6	6	107	2.81	46.7	2.6	106	5.38	2.22	.041	2.27	.178
6F¶	6	113	2.77	42.9	3.04	153	10.1	2.63	.032	3.62	.210
HeLa											
4	6	75.2	1.46	19.8	.72	86.3	1.46	3.79	.061	4.36	.21
7	6	64.1	.93	23.9	.70	78.7	2.94	2.64	.069	3.32	.14
7F	6	87.2	2.02	26.8	1.3	100	1.48	3.26	.008	3.75	.18
McCoy											
3	6	111	3.7	53.9	2.7	95	6.9	2.1	.054	1.93	.060
5	9	170	5.1	79.6	2.9	119	5.2	2.13	.039	1.61	.026
5F	8	240	3.2	99.5	2.7	157	4.6	2.42	.033	1.72	.022

* TPP is expressed in DNA equivalents. 10 μg of DNA/ml in acid salt solution has an absorbance of 0.260 at 268 mμ (1 cm light path).

† The reference protein was a purified preparation from HeLa cells.

‡ Avg values.

§ Stand. dev., calculated from difference between duplicate determinations for each culture, on the assumption that all cultures of a group were samples of the same population.

‖ Stand. error. Ratios were calculated for each culture individually. These values were used for calculation of mean ratio and stand. error of the mean.

¶ Letter F indicates that cultures were fed 24 hr before they were terminated.

ard is required with each experiment. 3. The TPP method has the much wider range of operation. Readings can be made very accurately, and there is a linear relationship between TPP and cell number between absorbances of 0.1 and 2.0.

Summary. This paper presents a new method for measuring populations of tissue cell cultures. The basis of this method is a simple extraction and the measurement of total purines and pyrimidines (TPP) by their absorbance at 268 mμ. A comparison of TPP/DNA to PN/DNA in 3 cell strains growing under different conditions shows that TPP is at least as good as PN for measuring cell populations.

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Biosynthesis of Hyaluronic Acid in Tissue Cultures of Human Synovial Membrane.* (23947)

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It has been demonstrated(1,2) that hyaluronic acid in synovial fluid from cases of rheumatoid arthritis had a lower intrinsic viscosity than that from normal persons. Similar changes in the quality of synovial hyaluronic acid have been shown by Ragan and Meyer(3), and were ascribed by them to synthesis of incompletely polymerized hyaluronic acid. Synthesis of acid mucopolysaccharides has been studied in tissue cultures from synovial membrane by Vaubel(4), Kling *et al.*(5), Grossfeld *et al.*(6) and Castor(7). The results suggested that hyaluronic acid was produced.

The purpose of the present investigation was to study biosynthesis of acid mucopolysaccharides in tissue cultures of human synovial membranes from noninflammatory cases, and to try to obtain information on the intrinsic viscosity of the mucopolysaccharides produced.

Materials and methods. Human synovial and periarticular connective tissue were obtained by meniscectomy from male, non-inflammatory cases. Pieces of tissue, removed at operation, were placed in Tyrode solution for $\frac{1}{2}$ to 1 hour. Before cultivation the synovial membrane was, as far as possible, freed from subsynovial tissue. The cultures were generally made in flasks of the Grand type using culture media described by Kling *et al.*(5). Streptomycin and penicillin—50 units/ml of each—were added. The fluid phase (5 ml) of the culture medium was changed once a week. As controls, nutrient media were incubated without cells (control medium, one control for each new batch of media).[†] Viscosity of the fluids was measured (after cen-

trifugation) at 37.0°-37.3°C according to Diczfalussy *et al.*(8). Ostwald viscometers (2 ml) with efflux times (for water) varying from 12 to 16 sec. were used. Relative viscosity (η_{rel}) = ratio between the efflux times for solution and solvent; specific viscosity (η_{spec}) = difference between (η_{rel}) for supernatants of cultures and the controls. Calculation of the error of the method from duplicate determinations showed that a difference in relative viscosity of 0.03 (= 3 x ϵ) was significant.

Determination of acid mucopolysaccharides. Samples were depolymerized by testicular hyaluronidase (100 VRU/ml) and deproteinized with trichloroacetic acid as described by Sundblad(1). Hexosamines were then determined according to Blix(9). The standard error of a single determination was calculated from double determinations to be $\pm 7.2\%$. The degree of interference by chromogens (10) (as compared with a solution of pure glucosamine-HCl) was so small that it could be neglected. In any case it was of no importance when the increase of hexosamines (= difference between culture medium and control medium) was reported. **Tests for characterization of viscosity increasing substance (s):** Samples were tested for mucin clot formation according to Grossfeld *et al.*(6). The viscosity reducing effect of testicular (Hyalas[®], AB Leo Hälsingborg, Sweden) hyaluronidase, purified by chromatography, and staphylococcal hyaluronidase (Hyason[®], N.V. Organon, Oss, Holland) was determined. Generally 78 VRU Hyason and up to 100 VRU Hyalas were used (all units expressed as units of Hyalas per 2 ml).[‡] Incubation was for 1 hour at 37.0°-37.3°C. Dialysis was per-

* We are indebted to colleagues in the Orthopedic departments of the Hospitals in Malmö and Lund (Sweden) for generously providing us with tissue material; and to AB Leo, Hälsingborg, Sweden, and N. V. Organon, Oss, Holland, for liberal supply of their hyaluronidase preparations.

This investigation was supported by research grants from Gustaf V 80-years Fm.

[†] Tissue cultivations were performed by Doc. B. Kallén at the Tissue Culture Laboratory, Inst. of Histology, Lund, Sweden.

[‡] Presence of considerable amounts of inhibitors (determined according to Adner(11)) in the fluids examined, necessitated the use of these high amounts of enzymes.

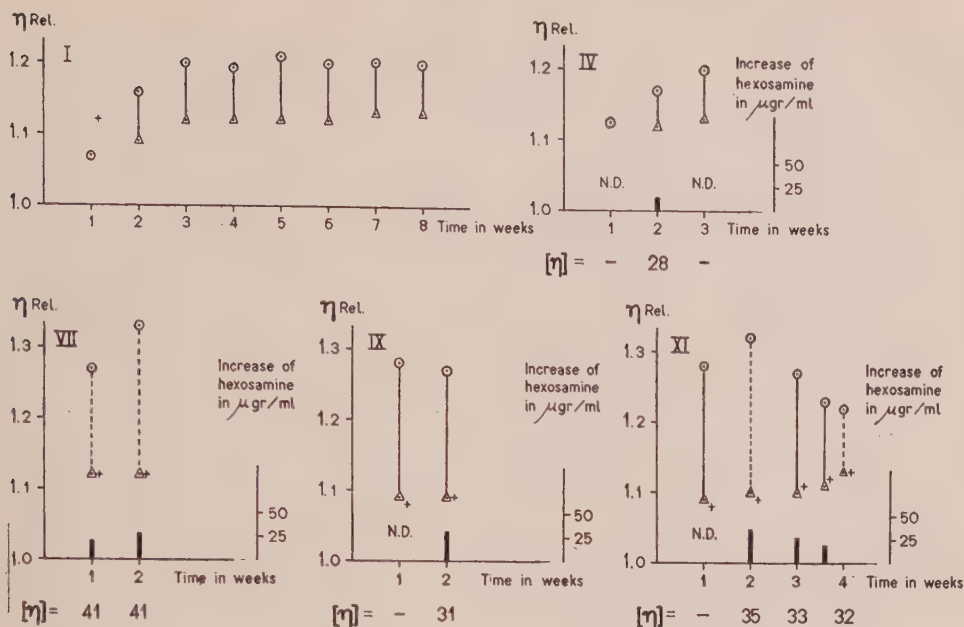


FIG. 1. Increase of viscosity and mucopolysaccharide-hexosamines in supernatants of tissue cultures from human synovial membrane. η Rel. = relative viscosity; $\bigcirc$ = relative viscosity before incubation with hyaluronidase; $\triangle$ = relative viscosity after incubation with hyaluronidase; | = viscosity reduction after incubation with testis hyaluronidase; + = viscosity reduction after incubation with staphyloc. hyaluronidase; + = relative viscosity of control medium; | = increase of mucopolysaccharide-hexosamines; $[\eta]$ = intrinsic viscosity.

formed (on several samples selected at random) either as rapid dialysis according to Mies(12) against isotonic buffer saline mixture (pH = 7.0) at room temperature, or as extensive dialysis at + 4°C for about 48 hours against large volumes buffer saline (changed 4 times) with cellophane sausage tubing (Visking Corp., Chicago). The intrinsic viscosity $[\eta]$ was calculated as the ratio between specific viscosity and hexosamine concentration (in g/100 ml) $\times 2.12$ (1:2.12 = the molar ratio between hexosamine and the repeating unit of hyaluronic acid).[§]

Results. In Table I are reported the results of tissue cultures, clinical material and pro-

TABLE I. Clinical Material (Non-inflammatory Cases with Lesions of the Meniscus) and Tissue Cultures.

Case No.	Age, yr	Duration of disease, yr	Tissue cultures *	Production of mucopolysacch.
I	32	11-12	+	+
II	49	1/3	Fibrinolysis	—
III	59	1/4	"	—
IV	21	1/3	+	+
V	?	?	Fibrinolysis	—
VI	?	?	"	—
VII	30	<1/12	+	+
VIII	72	2	Necrosis	—
IX	53	2	+	+
X	15	1/4	+	+
XI	31	1	+	+
XII	52	1/2	+	+

* + = cell growth.

duction of acid mucopolysaccharides. The cultures were checked for cell growth and in one case a time-lapse movie was taken for about 20 hours. No signs of mitosis in the synovial cells could be observed. The liquid medium from cultures of human synovial membrane was more viscous than the liquid medium incubated without cells (= control medium (Fig. 1)). In Fig. 2 the results from

[§] As shown in previous investigations a linear ratio existed between viscosity and concentration of hyaluronate preparations for values of the latter below 0.05% (1,13,14). In the present investigation this relationship was valid even for the increase of viscosity in supernatants from tissue cultures. Because of this linear ratio the intrinsic viscosity(1), defined as $\lim_{c \rightarrow 0} \frac{\eta_{\text{spec}}}{c}$ (c = concentration) could be calculated as the ratio $\frac{\eta_{\text{spec}}}{c}$.

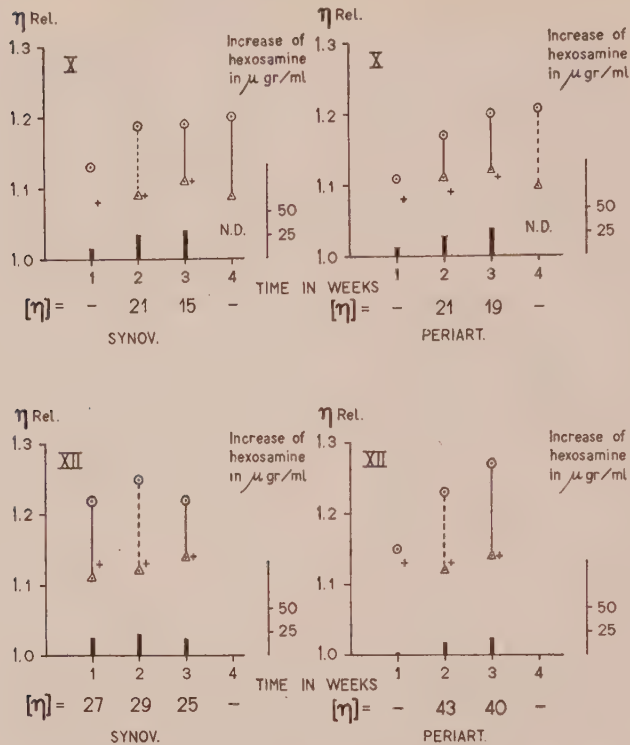


FIG. 2. Comparison between tissue cultures from human synovial membrane and from periarticular connective tissue. Symbols as in Fig. 1.

2 cultures of periarticular connective tissue are included. After 2 or 3 weeks, the increase of viscosity attained about the same level as in corresponding cultures from synovial membranes. The increase of viscosity could be reduced to viscosity of the control medium through incubation with testicular or staphylococcal hyaluronidase (see Fig. 1 and 2). It should be mentioned that the viscosity of the control medium could not in any case be reduced through incubation for at least one hour with testicular or staphylococcal hyaluronidase. Largely parallel with the increase of viscosity, there was an increase of acid mucopolysaccharides in the liquid medium (Fig. 1, 2). In addition, the mucin clot formation test, when performed, was positive in the liquid medium after cultivation and negative in the control medium.

To find out whether hexosamine containing oligosaccharides (which are dialysable) could be demonstrated in the liquid medium, some samples were dialysed. The results indicated that small amounts of oligosaccharides were

present before and after cultivation. In no case was there a significant increase of oligosaccharides after cultivation.

In tissue culture experiments, as performed, the amounts of tissue in the flasks varied, thus rendering comparisons between amounts of mucopolysaccharides produced impossible. However, an idea of their quality might be obtained from the intrinsic viscosity (see materials and methods). The numerical value of the intrinsic viscosity showed a considerable variation (15-43) from one case to another. With the exception of the synovial culture from case X, intrinsic viscosity seemed to be rather constant from week to week. Mucopolysaccharides of a synovial culture as compared with those of a periarticular origin showed about the same values of the intrinsic viscosity in case X and somewhat lower values in case XII (Fig. 2). When determining the hexosamine content of the mucopolysaccharides of the liquid medium not incubated with cells, considerable variations were found (13-34 γ mucopolysaccharide-hexosamine/

ml). In several cultures (7 out of 16) the amount of mucopolysaccharides was about 270% of that initially present in the liquid medium.

Discussion. Kling *et al.*(5) Grossfeld *et al.* (6) and Castor(7) performed quantitative determination of mucopolysaccharides (in supernatants of tissue cultures), by means of turbidimetric methods. The turbidimetric values of supernatants were related to a standard curve showing extinction values obtained from dilutions of a preparation of purified hyaluronic acid. Castor(7) compared the turbidimetric method with determinations of the hexosamine content of mucopolysaccharides (after dialysis), and found considerable differences: only 12-27% could be accounted for by turbidimetric determination of hexosamines. In the present study determination of the hexosamine content was performed for quantitative assay of acid mucopolysaccharides. Determination after dialysis of samples showed only a slight decrease of the hexosamine content in the control medium as well as in supernatants from tissue cultures. Thus production of large amounts of dialysable hexosamine containing substances, *i.e.* oligosaccharides, discussed by Castor(7) as a possible explanation of the differences found by him, could not be demonstrated in the present investigation. It should be observed that Castor(7) did not report any determinations of hexosamines *before* dialysis.

In the present study, the viscosity increasing non-dialysable substance(s), containing hexosamines and forming mucin clot could be depolymerized by testis hyaluronidase. These observations together, are in accordance with previous reports on presence of acid mucopolysaccharides in supernatants of tissue cultures from synovial membranes(4-7). As shown by Woodin(15) hyaluronic acid was depolymerized by staphylococcal hyaluronidase in contrast to chondroitin sulphate, while both these substrates were depolymerized by testis hyaluronidase. As shown in Fig. 1 and 2 the increase of viscosity could be completely eliminated through staphylococcal hyaluronidase. This observation indicates that *the increase of viscosity was caused by hyaluronic acid*.

Owing to low concentrations, errors of the methods used, and approximations implied in the calculation (all of the increase of mucopolysaccharide-hexosamines was calculated as hyaluronic acid), our values of intrinsic viscosity might be regarded as relative and semi-quantitative. Still these values are of the same order of magnitude as the *in vivo* values given by Sundblad(1) for hyaluronic acid of synovial origin. Provided the increase of mucopolysaccharides (see material and methods) reflected real production, the values of intrinsic viscosity thus found are compatible with the assumption that hyaluronic acid constituted the main part of acid mucopolysaccharides produced *in vitro*. The constancy of the intrinsic viscosity from week to week observed in the present study (Fig. 1, 2) suggested that as a principle the intrinsic viscosity might be used as a measure of quality of the mucopolysaccharides produced in tissue cultures.

In contrast to results published by Kling *et al.*(5), production of hyaluronic acid could be demonstrated even in cultures from periarticular connective tissue. The intrinsic viscosity of supernatants from these cultures seemed to show the same or a somewhat higher value if compared with the intrinsic viscosity in the corresponding synovial cultures.

Of interest is the question from what sources the acid mucopolysaccharides are synthesized. The increase of mucopolysaccharides in the supernatants of tissue cultures (main part of increase is believed to be produced by living cells) indicates that other sources than mucopolysaccharides of the nutrient medium are used in the synthesis. It has been shown by Yielding *et al.*(16), in their investigation on human synovial slices, that both the hexosamine and glucuronic acid parts of hyaluronic acid can be synthesized from glucose.

Summary. Production of acid mucopolysaccharides in tissue cultures from human non-inflammatory synovial membranes was studied. The results indicate that hyaluronic acid was the chief acid mucopolysaccharide produced. Hyaluronic acid was synthesized by the synovial as well as by the periarticular tissues. The results did not indicate an ap-

preciable production of (dialysable) hexosamine containing oligosaccharides. Intrinsic viscosity of the acid mucopolysaccharides produced varied from one case to another but kept rather constant from week to week.

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Relationship of Steroid and Pituitary Hormones to Myocardial Calcification in the Mouse.* (23948)

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Calcification of the myocardium can readily be induced in the mouse with hydrocortisone(1), and increases in blood calcium to toxic levels have been demonstrated in the dog following administration of the steroid (2). Furthermore, it is recognized that, in the human, hyperfunction of the adrenal glands is often accompanied by a negative calcium balance; if excessive production of corticoids is maintained for a prolonged period of time, as occurs in Cushing's syndrome, myocardial damage, calcific deposition, and osteoporosis may result(3). In an earlier study with intact C3H mice, it was established that the mineral deposit associated with the cardiac lesion is a calcium salt and that the degree of calcification is related to the dosage of hydrocortisone administered(1). The participation of other steroid and pituitary hormones in

this degenerative process is described here.

Procedure. Female mice of the C3H strain were used throughout the study, with the exception of a few males that were included in order to ascertain whether or not endogenous androgen would afford some protection to the heart. The mice were injected subcutaneously once daily with the hormones for 15 days, beginning at 50 days of age. Hypophysectomies were performed at 35 days of age, ovariectomies and adrenalectomies at 42 days of age. The adrenalectomized animals were given free access to 1% sodium chloride water and to tap water. The animals were fed a modified McCollum's Diet I, *ad libitum*(4), except for those whose sodium or potassium intake was restricted. At autopsy, the heart was inspected for grossly visible calcific deposits and the intensity of the calcification was estimated on an arbitrary scale of 0 to 5. In addition, the weights of the adrenals, heart and right kidney were recorded. The hydrocortisone, corticosterone, desoxycorticosterone (DOCA), testosterone, and estradiol were administered as

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TABLE I. Effect of Hydrocortisone, Corticosterone or Desoxycorticosterone on Myocardial Calcification in Female C3H Mice.

*	Treatment†	Daily dose, mg	No. of mice	Body wt, g		Organ wt, mg			Calcification‡
				Onset	Autopsy	Adrenals	R. kidney	Heart	
N	None	.0	6	18.5	20.5	3.7 ± .1§	113 ± 4	89 ± 2	0
A	"	.0	4	17.5	19		102 ± 9	89 ± 9	0
H	"	.0	4	12.5	13	1.5 ± .1	61 ± 3	50 ± 2	0
N	Hydrocortisone	.3	5	20	20	2.7 ± .1	176 ± 8	133 ± 5	1½
A	"	.3	6	18	19		153 ± 10	127 ± 4	½
H	"	.5	5	12	12	1.8 ± .2		53 ± 2	+ in 1 of 5
H	Corticosterone	2.0	5	12	15	1.7 ± .2	65 ± 3	47 ± 3	¼
A	Desoxycorticosterone	.3	6	17.5	22		131 ± 7	114 ± 5	0
A	Hydrocortisone + Desoxycorticosterone	.3 + .3	6	17.5	19		162 ± 4	121 ± 7	¼

* Operative condition: N = normal mice; A = mice adrenalectomized at 42 days; H = mice hypophysectomized at 35 days.

† Single daily subcut. inj. were begun at 50 days of age and continued for a period of 15 days, except in the case of corticosterone where they were continued for 28 days.

‡ This estimate represents extent of calcification observed grossly in the heart at autopsy.

+ in 1 of 5 indicates that a minute deposit was observed in one of the 5 experimental animals.

§ Mean ± stand. error.

saline suspensions.† The growth hormone was prepared from bovine pituitaries by the procedure previously described (5). The purified adrenocorticotrophic hormone fraction (designated as ACTH-F, and having an activity of 40 I.U. per mg) was prepared from sheep pituitaries and the α -corticotropin (α -ACTH, with an activity of 100-150 I.U. per mg) was isolated by a further purification of this material (6); ACTH preparations were administered in 16% gelatin. The Dowex 50-4X resin was prepared by being decanted and recycled in 2 N HCl and 0.5 N KOH.

Results. Calcification of the myocardium was consistently induced in intact female mice at daily dose levels of hydrocortisone above 0.25 mg. The intraventricular septum was the preferential site for deposition although as the calcification became more extensive, the right ventricle was also affected; only rarely was the left ventricle involved. The participation of an adrenal hormone other than hydrocortisone was not a prerequisite for the calcific deposition, for removal of the adrenal glands prior to injection of hydrocortisone did

not prevent the appearance of the myocardial lesions. Moreover, simultaneous administration of 0.50 mg of DOCA did not aggravate the condition (Table I).

In the absence of the pituitary gland, both hydrocortisone and ACTH were almost entirely ineffective in inducing calcification of the myocardium. In accord, however, with earlier observations that administration of growth hormone along with hydrocortisone or ACTH aggravates calcification in the intact mouse (1), it was demonstrated in this study that a similar enhancement is produced by growth hormone in the hypophysectomized animal (Table II). Contrariwise, lactogenic hormone given at a daily dose level of 0.10 mg along with ACTH gave results similar to those obtained with ACTH alone.

In the intact animal, hormones other than pituitary growth hormone, *e.g.*, the sex hormones, are probably operating to modify the cardiac calcification induced by hydrocortisone. The non-operated male was much less affected than the female; however, removal of the testes abolished this protective effect so that the myocardial condition in the castrated male was comparable to that in the intact female. In the female, the calcification was alleviated by administration of 0.50 mg of tes-

‡ Hydrocortisone acetate and corticosterone were obtained from Merck & Co.; testosterone, estradiol and desoxycorticosterone acetate were products of Schering Corp.

TABLE II. Effect of ACTH plus Growth Hormone or Lactogenic Hormone on Myocardial Calcification in Hypophysectomized Female C3H Mice.*

Treatment	Daily dose, mg	No. of mice	Body wt, g		Organ wt, mg			Calcification†
			Onset	Autopsy	Adrenals	R. kidney	Heart	
None	.0	4	12.5	13	1.5 ± .1‡	61 ± 3	50 ± 2	0
ACTH-F	.8	4	12	15	6.2 ± .2	99 ± 4	60 ± 2	+ in 4 of 4
α -corticotropin	.1	5	13	15	6.5 ± .2	92 ± 2	52 ± 1	+ in 1 of 5
Growth hormone	.1	10	12.5	19	2.2 ± .2			0
<i>Idem</i>	1.0	7	13	19	2.8 ± .3	100 ± 3	78 ± 4	0
Lactogenic hormone	.1	5	11.5	12	1.5 ± .2	58 ± 5	52 ± 5	0
ACTH-F	.8							
+	+	5	13	16	10 ± .4	144 ± 5	81 ± 2	¾
Growth hormone	.1							
	.8							
<i>Idem</i>	+	6	13	17	8.6 ± .5	160 ± 7	94 ± 2	½
	1.0							
ACTH-F	.8							
+	+	6	12	15.5	8.1 ± .5	123 ± 5	68 ± 3	+ in 2 of 6
Lactogenic hormone	.1							

* All animals were hypophysectomized at 35 days of age. Single daily subcut. inj. were begun at 50 days of age and continued for a period of 15 days.

† This estimate represents the extent of calcification observed grossly in the heart at autopsy. + in 4 of 4 means that a minute deposit was observed in 4 of the 4 experimental animals, etc.

‡ Mean ± stand. error.

tosterone in conjunction with the hydrocortisone (Table III). On the other hand, administration of estradiol did not aggravate the condition, and ovariectomy was ineffective in preventing the deposition.

Inasmuch as an excess of hydrocortisone would be expected to promote sodium retention along with potassium excretion, and since calcified lesions of the myocardium and other tissues have been observed in animals maintained on various abnormal diets including those low in potassium (7,8,9), an attempt was made to ascertain whether the condition would be alleviated by increasing the potassium uptake of the animals or by decreasing their sodium intake. Four groups of mice were placed on special diets for the period of 15 days during which they received the hormone; however, the animals derived no benefit from any of the dietary modifications (Table IV). Those given a diet deficient in both sodium and potassium lost 40% of their original body weight and were in poor condition by the time of autopsy. The animals given free access to additional potassium in the form of 0.5% KCl H₂O or as the potassium form of the Dowex 50 resin exhibited no signs of

toxicity; however, the cardiac calcification was nonetheless aggravated. Similar effects were noted with the sodium-deficient diet.

Hypertrophy of the kidney and of the heart was obtained with hydrocortisone in the intact, the ovariectomized and the adrenalectomized animals. Hypertrophy of the kidney was likewise observed in the hypophysectomized animals with ACTH as well as with growth hormone, the increases in the weight of the organ being additive when the two hormones were injected concurrently. Cardiac enlargement, however, did not parallel the kidney enlargement and an increase in the weight of the heart was observed only in the hypophysectomized animals treated with growth hormone, not in those treated with α -corticotropin, hydrocortisone or corticosterone. It can also be seen from Table II that the weights of the adrenal glands were considerably larger in the hypophysectomized mice injected with ACTH plus growth hormone than in those injected with ACTH alone. Thus, in this instance there is a synergism operative between the two hormones, in contrast to the simple additive effect on the kidney noted above.

TABLE III. Effect of Hydrocortisone plus Testosterone or Estradiol on Myocardial Calcification in C3H Mice.

Treatment*	Daily dose, mg	No. of mice and sex	Body wt, g		Organ wt, mg			Calcification†
			Onset	Autopsy	Adrenals	R. kidney	Heart	
Hydrocortisone	.3	5 ♂	23.5	24	1.7 ± .1‡	227 ± 14	126 ± 5	+ in 4 of 5
"	.3	5 ♀	20	20	2.7 ± .1	176 ± 8	133 ± 5	1½
"	.3	5 ♀ §	19	23	2.6 ± .2	179 ± 9	134 ± 6	+ in 5 of 5
"	.5	4 ♂	22.5	20	2.1 ± .1	209 ± 10	144 ± 6	¼
"	.5	5 ♂ §	20	16.5	2.2 ± .1	190 ± 9	132 ± 4	2½
"	.5	4 ♀ §	19	18	2.6 ± .3	195 ± 4	134 ± 7	2½
Hydrocortisone	.3							
+	+	6 ♂	23.5	24.5	1.8 ± .1	238 ± 9	128 ± 4	+ in 5 of 6
Testosterone	.5							
	.3							
<i>Idem</i>	+	3 ♀	20	21	2.2 ± .2	151 ± 7	119 ± 2	1½
	.1							
"	.3	5 ♀	20	18	2.1 ± .1	192 ± 11	114 ± 9	¼
	.5							
"	.3	5 ♀ §	19	21.5	2.2 ± .2	243 ± 16	140 ± 5	+ in 3 of 5
	+							
	.5							
Hydrocortisone	.3							
+	+	8 ♀	20.5	20.5	4.3 ± .2	173 ± 2	117 ± 3	2
Estradiol	.002							
	.5							
<i>Idem</i>	+	4 ♀ §	19	21	3.9 ± .1	183 ± 7	141 ± 7	2
	.01							

* Single daily subcut. inj. begun at 50 days of age and continued for 15 days.

† This estimate represents extent of calcification observed grossly in heart at autopsy. + in 4 of 5 indicates that a minute deposit was observed in 4 of 5 experimental animals, etc.

‡ Mean ± stand. error.

§ These animals were gonadectomized at 42 days of age, 8 days prior to first inj.

Discussion. Hydrocortisone administered for a period of 15 days consistently induced calcification of the myocardium in normal female mice. Neither experimental renal damage(10) nor unilateral nephrectomy(11) was resorted to in these studies. However, hypertrophy of the kidneys did result from the treatment and some derangement in kidney function undoubtedly was involved in the degenerative process. Similar results were evident in the hypophysectomized animals, in whom enlargement of the kidneys was observed following administration of either ACTH or growth hormone.

The anterior pituitary appears to influence the course of the myocardial calcification in other ways besides secreting the ACTH that stimulates the release of corticoids from the adrenal. Thus, the administration of growth hormone aggravates the cardiac condition in both the hypophysectomized and the intact mouse. On the other hand, the gonadotropins

must contribute to some alleviation of the condition, inasmuch as the presence of the testes in the male as well as the concurrent administration of testosterone along with hydrocortisone to the female, afforded considerable protection to the heart. Selye has likewise found that calcification resulting from the administration of dihydrotachysterol (AT-10) is reduced by concurrent treatment with methyltestosterone(12). There was no suggestion that the ovarian hormones are involved in the process whereby calcific deposition is induced by the action of hydrocortisone; ovariectomy of the female did not prevent the occurrence of the deposits and the injection of estradiol had no effect whatsoever on the condition.

Attempts made in the present investigation to prevent the cardiac damage induced with hydrocortisone by increasing the potassium intake or by decreasing the sodium intake, were unsuccessful. On the other hand, en-

TABLE IV. Effect of Alterations in Sodium-Potassium Content of Diet on Myocardial Calcification Produced in Female C3H Mice with Hydrocortisone.*

Diet	Daily dose, mg	No. of mice	Body wt, g		Organ wt, mg			Calcification†
			Onset	Autopsy	Adrenals	R. kidney	Heart	
Stock	.3	5	20	20.5	4.0 ± .1‡	186 ± 5	124 ± 2	1½
"	.5	9	17.5	19	2.5 ± .1	164 ± 5	119 ± 3	3½
Stock + .5% KCl H ₂ O	.5	10	16.5	18.5	2.8 ± .1	166 ± 6	138 ± 6	4
Stock + .3% resin§	.5	10	17	19.5	2.7 ± .1	163 ± 3	123 ± 5	4
Sodium-deficient	.3	9	21.5	16	2.9 ± .2	124 ± 4	100 ± 3	4
Sodium-potassium-deficient	.3	6	20	11.5	2.5 ± .3	136 ± 7	69 ± 3	1¼

* Single daily subcut. inj. of hydrocortisone were begun at 50 days of age and continued for a period of 15 days.

† This estimate represents extent of calcification observed grossly in the heart at autopsy.

‡ Mean ± stand. error.

§ 100 mg of Dowex 50 resin (50 mg K form, 50 mg H form) distributed in 30 g of stock diet—approximately 45 mg resin/70 g body wt/day.

|| Special diets purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.

larged adrenal glands were observed in the earlier studies in which the animals had been given a potassium-deficient diet(13,14) and it may well be that an excess of the adrenal corticoids, rather than a deficiency of the K ion *per se*, was largely responsible for the production of the necrotic and calcareous lesions.

Summary. Hydrocortisone administered at daily levels above 0.25 mg for 15 days consistently induced calcification of the myocardium in adolescent female mice. The male was less affected than the female and the concurrent administration of testosterone to the female alleviated the condition; ovariectomy afforded no protection to the heart. Both hydrocortisone and ACTH were quite ineffective in inducing the deposition of calcium in the heart of the hypophysectomized mouse. Moreover, the administration of growth hormone with hydrocortisone or ACTH aggravated this condition. Lactogenic hormone, DOCA and estradiol had no effect.

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Relation between Pituitary-Adrenal System and Excretion of Trypsin Inhibitor in Urine.* (23949)

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Various early investigators(1,2,3) have studied the trypsin inhibitor (T.I.) in human urine. They observed an increased excretion in cancer and infectious diseases. Dillard (4) resumed these investigations with modern methods in an effort to devise a cancer test, but found the increase of T.I. in urine to be too unspecific for neoplasms. It was accidentally found in this laboratory that urine from pregnant women contains large amounts of T.I. (Astrup and Storm, unpublished). We have since observed an increased excretion of T.I. during pregnancy, in artificial fever, in various infectious diseases and during gold therapy(5). Since stress probably is the common denominator in these different conditions, excretion of T.I. in urine could be a physiological response to stress. The present investigations were based upon this hypothesis.

Methods. 1. *The inhibitor* (T.I.) was estimated against trypsin on a casein substrate using the biuret reaction. *Reagents.* *Buffer I*; 0.1 M barbital sodium, pH 7.75 + NaCl to ionic strength 0.15. *Buffer II*; buffer I, containing 0.01 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Crystalline trypsin (24.4 A.U./g) was kindly provided by NOVO Laboratories, Copenhagen. Casein, 3% prepared after(6), but dissolved in buffer I. Biuret reagent, modified according to(7). 0.50 ml buffer II, containing 3.75 μg trypsin, is incubated at 37°C with 0.50 ml urine solution (appropriately diluted with buffer II). After 20 minutes, 2 ml casein solution is added, and the mixture incubated 40 minutes at 37°C, followed by addition of 1 ml 20% perchloric acid. The mixture is filtered after one hour and 2 ml of the filtrate is added to 1 ml 2 N NaOH and 3 ml biuret reagent. After 40 minutes incubation at 37°C, the optical density is determined at 545

m μ . In the blanks perchloric acid was added (after 20 minutes incubation of trypsin with urine dilution) before addition of the casein solution. Free trypsin is found by interpolation on a trypsin dilution curve, and bound trypsin calculated. Inhibition of trypsin follows a straight line until 85% inhibition, above which it deflects, indicating that the compound formed is dissociable. All measurements were performed on the straight part of curve, and a T.I. unit was defined as the amount which can inhibit one μg of crystalline trypsin (24.4 A.U./g). Further details of the method will be published elsewhere. 2. *Hormone determinations* were performed according to standard procedures(8) by the Hormone Department of the Statens Serum-institut through the kindness of Dr. Chr. Hamburger.

Results. *Effect of adrenocorticotrophic hormone.* In 4 normal men and 4 normal women from 20 to 40 years of age, and in a 55-year-old male patient with aplastic anemia, the total daily excretion of T.I. in urine before and during treatment with ACTH was estimated. The normal subjects were given 25 i.u. ACTH i.m. on 2 successive days. The patient received daily doses of ACTH i.m. for 2 weeks. Table I and Fig. 1 demonstrate that T.I. excretion increases considerably during ACTH administration.

Effect of cortisone and hydrocortisone was studied in 10 normal persons (age: 20 to 55 years). The hormones were given orally (as acetates) in doses of 100 mg for 3 consecutive days (cortisone) or for 2 days (hydrocortisone). Cortisone and hydrocortisone, like ACTH, increase excretion of T.I. many times. Fig. 2 shows a typical curve. In only one (apparently normal) person administration of cortisone was for no obvious reasons not followed by an increased T.I. excretion.

17-ketosteroids, 17-ketogenic steroids and T.I. in urine. Urine from a normal woman

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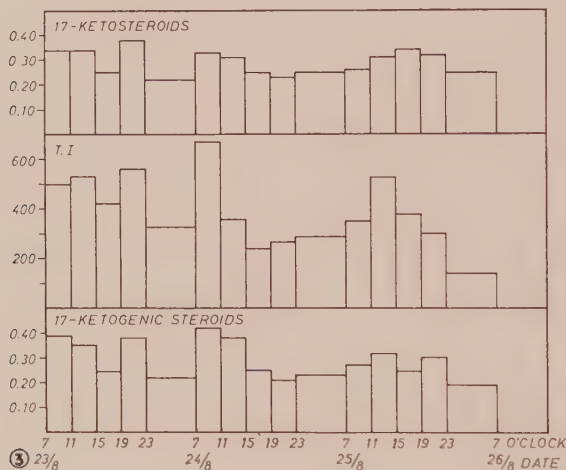
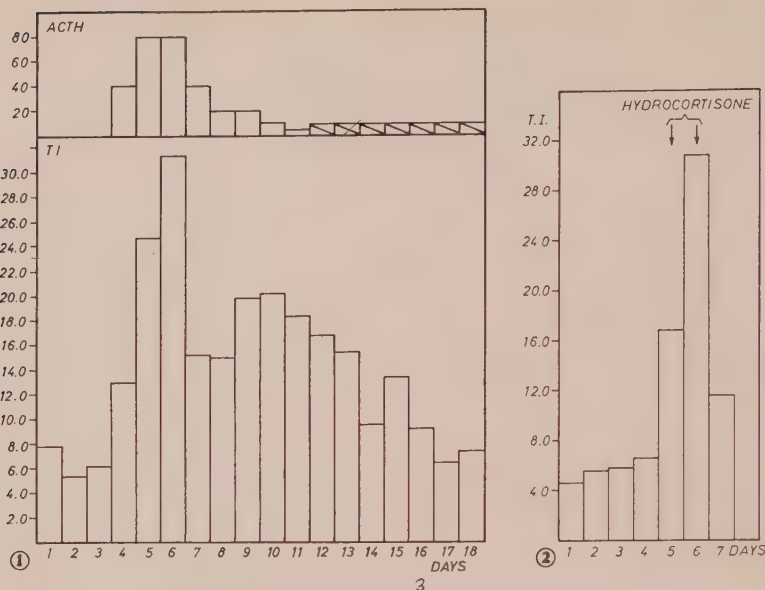


FIG. 1. Excretion of trypsin inhibitor in urine from a patient with aplastic anemia, before and during treatment with ACTH. *Abscissa*: Time in days. *Ordinates*: Trypsin inhibitor in units $\times 10^{-3}$, and dose of ACTH in international units. An ACTH preparation with protracted action was used in the last 7 days (columns marked with a diagonal).

FIG. 2. Effect of hydrocortisone on excretion of trypsin inhibitor. *Abcissa*: Days. *Ordinate*: Trypsin inhibitor in units $\times 10^{-3}$. The arrows indicate the days on which hydrocortisone acetate (20 mg 5 times daily) was given.

FIG. 3. Excretion of 17-ketosteroids, 17-ketogenic steroids and trypsin inhibitor in a woman. *Abscissa*: Date and hour. *Ordinates*: 17-ketosteroids and 17-ketogenic steroids in mg/hr. Trypsin inhibitor in units/hr.

was collected in samples every 4 hours during the day and once a night in 3 successive days. 17-ketosteroids, 17-ketogenic steroids and T.I. were estimated and the average excretion/hour calculated for individual samples. Fig. 3 shows that T.I. closely follows the steroid excretion.

Discussion. Since cortisone and hydrocortisone produce an increase of T.I. in urine, the increased excretion of T.I. in stress and during ACTH administration could be caused by the increased release of adrenocortical hormones in these conditions. The relation observed between the excretion of T.I. and of

TABLE I. Effect of ACTH on Excretion of Trypsin Inhibitor in 8 Normal Subjects.

Age, yr	T.I. units $\times 10^{-3}$ excreted					Avg T.I. excretion/day		Increase during ACTH treatment, %
	No treatment			ACTH, 25 i.u. i.m.		Before ACTH treatment	During ACTH treatment	
	Day 1	Day 2	Day 3	Day 4	Day 5			
30	.0	.0	.36	3.15	7.46	.12	5.31	4325
30	1.35	.90	.0	1.52	3.96	.75	2.79	272
30	.68	1.10	1.40	2.03	2.40	1.06	2.22	109
22	2.02	1.20	.90	4.14	4.71	1.37	4.43	223
40	3.10	2.88	1.51	5.11	11.79	2.50	8.45	238
29	4.73	4.04	7.96	13.73	16.77	5.58	15.25	174
24	7.80	5.07	5.70	16.17	29.04	6.19	22.61	265
40	10.54	5.98	7.35	15.39	20.68	7.96	18.04	127

17-ketosteroids and 17-ketogenic steroids in urine suggests a close connection between function of pituitary-adrenal system and excretion of trypsin inhibitor. The increase in T.I. could be caused either by increased production of inhibitor or by increased renal excretion. Though it is too early to consider any connections between the observations here presented and pathological states in the organism, it is interesting to note that some investigators have reported proteolytic activity in tissues and blood in conditions where ACTH and corticosteroids have a therapeutic effect, especially in anaphylactic reactions. The results support our hypothesis that the increase of trypsin inhibitor in urine in various physiological and pathological conditions is caused by a stress reaction.

Summary. 1. Administration of ACTH, cortisone or hydrocortisone to human beings produces an increase in excretion of trypsin

inhibitor in urine. 2. Excretion of trypsin inhibitor during the day is nearly parallel to excretion of 17-ketosteroids and 17-ketogenic steroids. 3. The pituitary-adrenal system plays a role in regulation of excretion of trypsin inhibitor.

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Nutritional Studies of Hydroxyzine and Rauwolfia in Cattle and Lambs. (23950)

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(Introduced by S. Y. P'An)

Chas. Pfizer & Co., Terre Haute, Ind.

Although tranquilizing drugs have been studied with monogastric animals, the levels of the drugs fed were within the sedating range. No reports of investigations of nutritional effects of tranquilizing drugs upon ruminants have been found. The present report

compares effects upon growth, feed efficiency and carcass quality of tranquilizing drugs fed to beef steers and wether lambs in a range generally below the level at which sedation would be expected. The tranquilizers tested were crystalline pharmaceutical grade hy-

TABLE I. Experimental Rations.

Ration No.	Cattle		Lambs*
	1	2	3
Corn + cob meal	Self-fed	Self-fed	
Alfalfa hay/day, lb		4	
Supplement/day, "	2	2	Self-fed
Mineralized salt	Self-fed	Self-fed	"
Composition of supplement	%		
Yellow corn meal			25.0
Soybean oil "	58.9	82.8	
Dehydrated alfalfa meal	20.0	20.0	
Sun cured alfalfa			64.9
Dried molasses	10.0	10.0	
Liquid "			10.0
Urea	5.0	3.0	
Dicalcium phosphate	2.0	4.0	
Ground limestone	1.75		
Trace mineralized salt	2.25		
Stabilized vit. A†	.1	.2	.1
Total	100.0	100.0	100.0

* The lamb ration was made into $\frac{3}{8}$ inch pellets and self-fed.

† Gelatin stabilized vit. A mix containing 5,000 U.S.P. units of vit. A palmitate/g.

droxyzine, 1-(p-chlorobenzhydryl - 4 - (2-hydroxyethoxy-ethyl)-diethylenediamine dihydrochloride, and ground *Rauwolfia vomitoria* root containing 0.24% reserpine, trimethoxybenzoylmethyl reserpate.

Procedure. Forty-one yearling Hereford steers and 108 whiteface cross-bred Texas wether lambs were employed as test animals. The basal rations for the animals in the different experiments are given in Table I. Feed consumption records were kept for each group of animals and individual body weights were taken for the steers and lambs every 28 and 14 days, respectively. In Trial 1, which lasted 84 days, *Rauwolfia vomitoria* was fed to groups of 4 steers each at levels of 5, 25 and 125 mg per head per day. An additional group of 4 animals which received the basal ration served as controls. In Trial 2, which lasted 111 days, hydroxyzine was given to groups of 4 steers each at levels of 1.25, 2.5, 10, 50 and 500 mg per head per day. A group of 5 comparable animals served as controls. All steers in Trial 2, including controls, were initially implanted with 36 mg diethylstilbestrol. In Trial 3, which lasted 69 days, 2 replicate groups of 12 lambs served as negative controls. Two additional replicate groups of 12 each were implanted with 50 and 3 mg, respectively, of oxytetracycline* and stilbes-

trol and served as controls for the tranquilizer treatments. Groups of 12 lambs each received the oxytetracycline-stilbestrol implants and were fed rations containing hydroxyzine at levels of 0.24, 1.2, 6.0 and 30 g per ton, respectively. An additional group of 12 lambs were fed on diets containing 3 g per ton of *Rauwolfia vomitoria*. These animals were also implanted with oxytetracycline-stilbestrol.

Results. The feed efficiency, dressing per cent, carcass grade and average daily weight gains of steers fed rations containing *Rauwolfia vomitoria* or hydroxyzine are given in Table II. In Trial 1 all levels of *Rauwolfia vomitoria* improved the daily weight gains. The best growth effect was found in the group receiving the 25 mg level. It showed a gain of 16% over the controls. The feed efficiency with all 3 levels of *Rauwolfia* was slightly improved over that of the controls. No effect upon the dressing per cent or carcass grade was apparent in these animals. In Trial 2, hydroxyzine improved both the rate of gain and feed efficiency of steers over the entire range of feeding levels. However, no consistent dose-response relationship was apparent with either of these phenomena. Although the carcass grade of the 2.5 mg level suggested a possible improvement, it remained practically unchanged with virtually all the other levels. Because of the limited number of steers in the various groups in Trials 1 and 2 differences in the average daily gains were not statistically significant at the 5% level but approached significance at the 10% level and when all levels of hydroxyzine were compared with the control the difference was significant at the 10% level. The dressing percentages of the groups which received 1.25 and 2.5 mg hydroxyzine in Trial 2 were significantly better than the control at the 10% level.

The results of Trial 3 in which tranquilizing drugs were fed to lambs are given in Table III. The lambs with stilbestrol-oxytetracycline implants made significantly greater weight gains than the negative controls and feed efficiency was improved. Neither the dressing percentage nor the carcass grade was significantly altered. With the exception of the lowest levels, all hydroxyzine

* Terramycin, Chas. Pfizer & Co., Inc.

TABLE II. Response of Beef Cattle (Steers) to Oral Hydroxyzine and *Rauwolfia vomitoria*.

Supplement	No. of animals	Avg daily gain, lb	Lb feed per lb gain	Dressing, %	Carcass grade*
<i>Trial 1, 84-day results</i>					
Control	4	2.77	8.38	62.0	10.00
+ <i>Rauwolfia vomitoria</i> , 5 mg†	4	2.95	8.18	62.0	9.50
+ " " 25	4	3.21	8.12	62.0	10.25
+ " " 125	4	2.81	8.30	61.0	9.50
Avg, all <i>Rauwolfia</i> levels		2.99	8.20	61.7	9.75
Least significant difference ($P < .10$)					
Each treatment		.46			.79
All <i>Rauwolfia</i> treatments combined		.37			.65
<i>Trial 2, 111-day results</i>					
Stilbestrol implant, 36 mg	5	2.25	10.41	59.4	10.40
+ Hydroxyzine, 1.25	4	2.83	9.12	61.2†	10.25
+ " 2.5	4	2.68	9.16	61.1†	11.25
+ " 10	4	2.82	9.16	60.1	10.25
+ " 50	4	2.74	8.68	59.8	10.50
+ " 500	4	2.61	9.16	59.2	10.33
Avg, all hydroxyzine levels		2.74†	9.06	60.2	10.52
Least significant difference ($P < .10$)					
Each treatment		.60		1.35	1.80
All hydroxyzine treatments combined		.44		1.26	1.40

* Carcass grade numerical rating: 11 avg choice; 8 avg good.

† Reserpine levels supplied by 5, 25 and 125 mg of *Rauwolfia vomitoria* were respectively 12, 60 and 300 μ g.‡ Difference from control statistically significant at $P < .10$.

fed lambs showed an increase in weight gain over the estrogen and antibiotic implanted controls. The best weight gain and feed efficiency were seen in lambs fed the ration containing 1.2 g of hydroxyzine per ton. The 15% increased gains with this level were statistically significant at ($P < .05$). Best feed efficiency of the experiment also occurred in the group receiving 1.2 g per ton hydroxyzine. Dressing percentage did not appear to be affected by hydroxyzine except at the 30.0 g per

ton level where it was slightly lower. There was evidence of improvement in grade with 1.2, 6.0 and 30.0 g per ton hydroxyzine. Significant growth responses were obtained with *Rauwolfia* but feed efficiency was not equal to that with 1.2 g per ton hydroxyzine. There was also indication of lower dressing percentage and slight downgrading in the *Rauwolfia* fed group.

Discussion. No evidence is immediately available to explain the mechanism by which

TABLE III. Response of Lambs (Wethers) to Oral Hydroxyzine and *Rauwolfia vomitoria* (Trial 3-69 Days).

Supplement	No. of animals	Avg daily gain, lb	Lb feed per lb gain	Dressing, %	Carcass grade*
Control	24	.370	9.48	47.3	7.9
Stilbestrol impl., 3 mg + oxytet. impl., 50 mg	24	.477†	7.35	46.0	8.0
+ Hydroxyzine, .24 g/ton	12	.473	7.54	45.7	7.5
+ " 1.2	12	.547†	6.98	45.6	8.2
+ " 6.0	12	.498	7.17	45.7	8.2
+ " 30.0	12	.485	7.31	44.8	8.3
+ <i>Rauwolfia vom.</i> , 3.0†	12	.551†	7.16	44.8	7.8
Least significant difference ($P < .05$)		.070		3.7	.7

* Carcass grade numerical rating: 11 avg choice; 8 avg good.

† The 3 g of *Rauwolfia vomitoria* contained 7.6 mg of reserpine.‡ Difference from appropriate control statistically significant at $P < .05$.

these tranquilizing drugs at low levels produce growth and feed efficiency responses. The obvious reasoning that the drugs decreased physical activity and consequently lowered energy expenditure without decreasing feed intake is not sustained. Frequent observations made on the degree of activity of these animals and ease of handling failed to produce evidence that any of the levels of hydroxyzine or *Rauwolfia vomitoria* fed to steers and lambs had produced any gross evidence of sedation. The doses which produced the best growth and feed efficiency responses in these experiments were at least 25 to 50 times below those recommended for therapy as tranquilizers. It is possible that minute doses of tranquilizing drugs exert a protective effect against environmental stress which does not present itself in the animals' gross behavior or otherwise. Reserpine, an active constituent of *Rauwolfia vomitoria* is known to counteract stress-induced depression of operant behavior using feed as a reinforcement(1). In contrast to this, the food intake in untrained rats was either unchanged(2) or depressed(3) following higher doses. This phenomenon, therefore, appears to be dependent on dose and possibly species.

The direct effects of these drugs on metabolic process as well as its influence on central mechanism controlling growth have also to be considered. The present interpretation of the mode of action of tranquilizing drugs in general points toward a specific effect of these compounds on the central nervous system(4), especially the brain stem. Brodie and Shore(5) postulated either release or blockade of specific neurohumoral agents to be an essential part of tranquilization, thus shifting the equilibrium of the autonomic balance from

the ergotropic (sympathetic) towards the trophotropic (parasympathetic) component (6). Such a mechanism of action might serve as a working hypothesis for the growth effects observed.

The importance and practical value of the observations reported here are obvious. The improvement in feed efficiency associated with growth promotion as a result of feeding tranquilizing drugs could have considerable economic importance. Extensive field tests, however, are essential before a final appraisal can be made.

Summary. 1. *Rauwolfia vomitoria* root fed to cattle at levels of 5, 25 and 125 mg per head per day improved growth rate and feed efficiency. Best results were obtained with the 25 mg level. Hydroxyzine fed to cattle at levels of 1.25, 2.5, 10, 50 and 500 mg per head per day improved growth with best results at 1.25 and 10 mg per head per day.

2. Wether lambs fed rations containing hydroxyzine at levels of 0.24, 1.2, 6.0 and 30.0 g per ton, or *Rauwolfia*, 3.0 g per ton, responded to all supplements. Optimum growth was obtained with a level of as low as 1.2 g per ton of hydroxyzine and 3.0 g per ton of *Rauwolfia*.

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Effect of Carbon Blacks on Carcinogenic Compounds.*† (23951)

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Von Haam(1) reported briefly on production of 6 epitheliomas in a total of 212 animals with benzene extracts of carbon blacks employed in the rubber industry: The active fractions were obtained by Scholl and Brock employing a procedure based on chromatographic separation and differential solubility. Subsequently, it was suggested that the apparent carcinogenic activity would probably be insignificant in the original carbon blacks because the isolated substances would be not only too low in concentration, but also firmly adsorbed(2). At about the same time Falk and Steiner(3) isolated 3,4 benzpyrene, 1,2 benzpyrene, pyrene and chrysene from benzene extracts of rubber stoppers and one old automobile tire. In later reports(4,5) they stated that benzene extracts of some carbon blacks contained small quantities of 3,4 benzpyrene, 1,2 benzpyrene and 5 other related aromatic hydrocarbons. They found a rough correlation between amounts of extractable aromatic hydrocarbons and average particle diameter of the carbon blacks and suggested that the difference in extractable aromatic hydrocarbons from carbon blacks may be explained by the phenomenon of adsorption. Despite the demonstrated presence of benzpyrene traces in some carbon blacks by two independent laboratories, Ingalls(6) found no evidence of environmental cancer among carbon black workers. In this communication are the results of *in vitro* and *in vivo* investigations on 7 carbon black preparations and their effect on carcinogenicity of 20-methylcholanthrene, 3,4 benzpyrene and p-dimethylaminoazobenzene.

Methods and materials. Adsorption procedure. Exactly 200 mg amounts of the carcinogenic substance in saturated cyclohexane solution were added to 20, 10, 5, 2.5, 0.625

and 0.313 g carbon black. The volumes were each brought up to 150 ml with cyclohexane. Each suspension was shaken mechanically for 2 hours and then filtered through a #1 Whatman paper into a 2 liter volumetric flask. The filter residues were washed with cyclohexane up to volume. The optical densities of the resulting filtrates were obtained at 360 m μ for 20-methylcholanthrene, 385 m μ for 3,4 benzpyrene and 410 m μ for p-dimethylaminoazobenzene: C.P. cyclohexane was the optical reference and 1.0 cm quartz cells were used throughout. Optical densities were also obtained on filtrates obtained by the identical procedure, except that the carcinogen was omitted. Moreover, for each series of unknowns, the optical densities of standard solutions of carcinogenic substances were also determined. *In vivo experiments.* Harlan stock rats and albino Swiss mice were used. The animals were kept in air-conditioned rooms and were fed a standard diet of Purina Dog Checkers with weekly additions of carrots and green vegetables except those in the azo dye experiments who received a pure rice diet since this has been shown to be most effective in production of neoplasms(7). For each carcinogenic substance, identical dosages were given in the free and adsorbed states. In addition an equivalent amount of carbon black without the carcinogenic substances was also administered. Thus, 3,4 benzpyrene was applied by painting the skin of mice twice weekly with acetone solution of the carcinogen at 0.5% concentration to one group; an acetone suspension of the filter residue containing the carcinogen adsorbed to carbon black to a second group; and a pure carbon black suspension in the same solvent to a third group. In another series of experiments, 20-methylcholanthrene either in the unadsorbed or the adsorbed state was injected subcutaneously into each rat in an approximate amount of 2 mg suspended in 1 ml of olive oil(8). P-dimethylaminoazobenzene was mixed with polished rice to give an approximate concen-

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TABLE I. Adsorption of Carcinogenic Compounds by Various Carbon Blacks.

Designation	Carbon black				mg carcinogen adsorbed by 1 g carbon black		
	Properties				20-methyl- cholanthrene	3,4 benz- pyrene	p-dimethyl- aminoazo- benzene
	Total surface*	E.M. surface†	Diameter, m μ	pH			
NeoSpectra Mark II	40	22	13	3.0	111.8	86.4	47.5
Peerless	20	12	24	2.8	40.4	41.3	27.5
Micronex-deactivated	10	10	29	10.5	17.0	12.2	9.5
Kosmos 60	11	11	27	8.9	10.9	11.7	6.7
Micronex beads	10	10	29	4.5	9.6	8.8	7.2
Philblack O	10	10	29	9.0	9.7	9.7	4.9
Micronex	10	10	29	4.5	8.3	7.5	3.3

* Total surface values in acres/lb are based on iodine adsorption measurements.

† E.M. surface values in acres/lb are based on electron microscope measurements, assuming smooth particle surfaces.

tration of .06% and the animals were fed *ad libitum*. The azo dye adsorbed to carbon black was mixed with food to produce an equal concentration (0.06%). All the animals were sacrificed at the end of the experiment and a complete autopsy was performed on each animal in order to determine any effect the carbon black itself had upon the organism. The application sites as well as all organs including the central nervous system were examined histologically.

Results. Table I lists the mg methylcholanthrene, p-dimethylaminoazobenzene and benzpyrene adsorbed by 1 g carbon black as related to the physical properties of the 7 carbon blacks. It is apparent that NeoSpectra Mark II has the highest adsorption property followed by Peerless and Micronex deactivated. The least adsorption was found with Micronex. As far as the carcinogenic compounds are concerned, the amounts of 3,4 benzpyrene and 20-methylcholanthrene were almost equal and approximately twice that of p-dimethylaminoazobenzene. Because of the low adsorption of azobenzene to certain carbon blacks, the data on Philblack O and Micronex are estimations at best since they were obtained by considerable extensions of experimental curves. Moreover, the optical densities of the filtrates plus washings of all 7 carbon blacks in the control experiments were 0.00 or identical with that of C.P. cyclohexane, indicating the absence of substances absorbing light at 360, 385 and 410 m μ .

The results obtained from the *in vivo* adsorption study with 20-methylcholanthrene are summarized in Table II. In the positive

control group using 20-methylcholanthrene in olive oil, 54% of the animals showed tumors 4 to 8 months after initiation of the experiment. The tumors proved to be highly undifferentiated spindle-cell sarcomas. The control animals injected with carbon black (Philblack O, 210 mg; Kosmos 60, 185 mg; Micronex Beads, 240 mg) did not develop any tumor in 12 months. Animals injected with 20-methylcholanthrene adsorbed on these carbon blacks developed tumors approximately at the same time as the control animals. Those injected with 20-methylcholanthrene adsorbed to Micronex Beads, developed tumors much later. Since these data suggested that adsorption to carbon blacks does not interfere appreciably with the carcinogenicity of 20-methylcholanthrene with the possible exception of that adsorbed to Micronex Beads, an additional experiment was carried out in which this carcinogen adsorbed to carbon black in 20% olive oil suspension was allowed to stand at room temperature in a sealed container. In 4 weeks, approximately one-fifth of the carcinogen was eluted by the olive oil.

The data from the *in vivo* experiments with p-dimethylaminoazobenzene are also presented in Table II. The nearly perfect inhibitory effect of adsorption on the carcinogenicity of butter yellow is broken only by a single hepatoma which appeared 10¼ months after the beginning of the experiment in one animal fed butter yellow adsorbed to Philblack O. None of the other animals have shown any evidence of neoplasm in spite of the common occurrence of liver cirrhosis. The

TABLE II. Effect of Carbon Blacks on Carcinogenicity of 20-Methylcholanthrene, p-Dimethylaminoazobenzene and 3,4 Benzpyrene.

Substances administered		Total No. animals	%	Results	
Carcinogen	Carbon black			Interval (mo)	
			tumors	First tumor	50% of tumors
Methylcholanthrene	None	24	54	4	6½
None	Philblack 0	24	0	0	0
Methylcholanthrene	<i>Idem</i>	24	29	4	6½
None	Kosmos 60	24	0	0	0
Methylcholanthrene	<i>Idem</i>	24	66	3½	5
None	Micronex beads	24	0	0	0
Methylcholanthrene	<i>Idem</i>	24	16	7	8½
Azobenzene	None	24	58	6	9
None	Philblack 0	24	0	0	0
Azobenzene	<i>Idem</i>	24	4	10¼	—
None	Micronex	24	0	0	0
Azobenzene	<i>Idem</i>	24	0	0	0
None	Kosmos 60	24	0	0	0
Azobenzene	<i>Idem</i>	24	0	0	0
Benzpyrene	None	48	96	2	3½
None	NeoSpectra Mark II	48	0	0	0
Benzpyrene	<i>Idem</i>	48	69	3	5¼
None	Peerless	48	0	0	0
Benzpyrene	<i>Idem</i>	48	63	3	6
None	Kosmos 60	48	0	0	0
Benzpyrene	<i>Idem</i>	48	59	3½	6
None	Micronex	48	0	0	0
Benzpyrene	<i>Idem</i>	48	54	3½	5
None	Micronex-deactivated	48	0	0	0
Benzpyrene	<i>Idem</i>	48	54	3¼	5

number of survivors in all but the positive control series is still considerable (42%) 15 months after initiation of the experiment. The animals ate without difficulty food containing the following concentrations of carbon black: Philblack O, 12%; Micronex, 18%; and Kosmos 60, 9%.

The effect of carbon black adsorption on carcinogenicity of 3,4 benzpyrene is summarized in Table II. In the positive control group 96% of the animals developed fatal carcinoma. None of the 240 animals which served as carbon black controls developed any tumor. The acetone suspensions contained from 6 to 60% carbon black. Of all the 240 animals treated with carbon black-adsorbed 3,4 benzpyrene suspended in a solvent, 54 to 69% developed tumors, the earliest of which appeared 3 months after the beginning of the experiment, a delay of one month as compared with the positive controls. Duration of the benzpyrene experiments was 9 months and number of survivors ranged

from 33 to 57%. Analyses of the supernate obtained from the acetone suspended material demonstrated that 10.0-23.4% of the adsorbed compound was eluted by this solvent and therefore acted as free 3,4 benzpyrene. In an attempt to circumvent this problem of elution, the adsorbed 3,4 benzpyrene without solvent was rubbed on the skin of 72 mice twice weekly: At the end of 24 months, no tumors were observed in this group as well as in 72 mice receiving carbon black alone. However, in 24 positive control mice treated with the dry carcinogen alone, only 12% of the animals developed tumors in contrast to 96% when 0.5% benzpyrene in acetone was employed.

Discussion. The results of the physical experiments conclusively demonstrate that carbon blacks adsorb 3 well-known carcinogenic compounds and that degree of adsorption depends on type of carbon black. There was a close parallelism between total surface values of the various carbon blacks and amounts of

20-methylcholanthrene, 3,4 benzpyrene and p-dimethylaminoazobenzene adsorbed. It would also appear from examination of the data obtained on the 3 varieties of Micronex that excessively high pH, such as 10.5, is another factor. The 2 pentacyclic hydrocarbons had twice the affinity to carbon blacks than the azo compound.

The data indicate that adsorption to carbon blacks has a varied but definite inhibitory effect on the carcinogenicity of the 3 compounds investigated. The almost complete reduction in the carcinogenic activity of azo dye by carbon blacks was just as pronounced as that reported by Hanszen and Selle(9). These investigators fed montmorillonite adsorbed dye to 20 animals for 175 days and only one developed a tumor as compared to 18 of the 20 control rats. The effect of the various carbon blacks on the carcinogenicity of 20-methylcholanthrene varied widely. Micronex Beads significantly reduced the number of tumors and delayed their appearance while Kosmos 60 was not inhibitory. A probable explanation for the latter results is associated with the finding that the vehicle, olive oil, elutes 20-methylcholanthrene which had been adsorbed on carbon black. Similarly, elution must also be considered in the 3,4-benzpyrene experiments, in which although the number of tumors decreased and their appearance delayed there was incomplete inhibition of carcinogenicity by carbon black. The acetone eluted material had a concentration of 0.10 to 0.23% free 3,4 benzpyrene, which according to Crabtree(10) can be carcinogenic.

Another interesting fact arising from these experiments is that carbon black, *per se*, either fed, painted with an acetone suspension or injected subcutaneously in olive oil suspension has not produced a single benign or malignant tumor. It must be noted that the carcinogenic substances or activity previously reported(1-4) were found in fractions obtained by exhaustive extraction, separation and concentration procedures on carbon black alone or on carbon black containing products

such as rubber stoppers or tires.

Summary. 1. Adsorption of 20-methylcholanthrene, 3,4 benzpyrene and p-dimethylaminoazobenzene to 7 different carbon blacks has been determined quantitatively. Differences in the amount adsorbed are related not only to the specific carcinogen but also to the total surface value of a carbon black. 2. Administration of carbon black alone by feeding, painting and subcutaneous injection did not produce any benign or malignant tumor in 460 animals. 3. The carcinogenicity of p-dimethylaminoazobenzene was almost completely inhibited by carbon black. Only 1 out of 72 animals fed carbon black adsorbed azo dye developed a hepatoma. 4. Administration of carbon black adsorbed 20-methylcholanthrene and 3,4-benzpyrene in combination with olive oil and acetone respectively inhibited carcinogenesis to a lesser degree. The incidence of tumors occurring in these experiments was approximately that expected from the concentrations of the free carcinogen eluted by the vehicle from the carbon black. 5. It is therefore suggested that in the adsorbed state all 3 compounds tested are not carcinogenic and that carcinogenicity in two of the compounds was restored due to elution by either olive oil or acetone.

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Production of Portal Hypertension and Esophageal Varices in the Mouse. (23952)

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(Introduced by T. von Brand)

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Taylor(1) has recently reviewed the field of experimental portal hypertension. He stated, seconded by Child(2,3), that the "enigma of portal hypertension is at present complete." He further noted that, "It is likely to remain so until an acceptable experimental method of producing it can be found." His major criterion for portal hypertension was a significant and sustained rise of portal pressure. Esophageal varices, if present, would not only simulate the consequences of portal hypertension in humans, but would indicate a sustained rise in pressure.

The purpose of this communication is to report the occurrence of portal hypertension and esophageal varices in mice experimentally infected with *Schistosoma mansoni*.

Materials and methods. Young adult Swiss albino mice were individually exposed to 100 cercariae of the Puerto Rican strain of *S. mansoni* according to the method of Olivier and Stirewalt(4). This produced an average of 24 worms in the portal circulatory system/mouse. The mice were sacrificed at two-week intervals from 6 to 16th week after infection. Portal pressure was measured by introducing a 22 gauge needle, connected to a strain gauge, into the portal vein. X-rays were obtained, under the same conditions, during injection of an

opaque medium (50% Hypaque) into the portal vein. Although esophageal varices when present were readily apparent at autopsy, they were preserved for anatomic and photographic study by injection of a yellow latex medium into a mesenteric vein at uniform pressure of 200 mm of mercury.

Results. Esophageal varices were observed in 43% of the mice examined from 10 to 16 weeks after infection. No varices were seen in control animals or in animals infected for less than 10 weeks. The varices arose from the venous plexus about the stomach, spleen, and liver and joined the systemic circulation, usually *via* the inferior vena cava or pulmonary veins, as was shown in latex injected preparations (Fig. 1) and X-ray portagrams (Fig. 2). The varices were uniformly on the serosal surface of the esophagus and were never within the esophagus itself. The average portal pressure in control animals was 5 cm of water (range 2-8). In infected mice the portal pressure, as shown in Table I, rose to an average of 10.3 cm (range 6-18.5) from 8th through 16th week (biweekly differences were not significant). From the tenth week on, when varices were present, the average portal pressure in 23 infected mice without varices was 9 cm of water (range 6-13.5) as

TABLE I. Portal Hypertension and Esophageal Varices in Mice Infected with *Schistosoma mansoni*.

	Wk after infection	Esophageal varices		Portal pressure			
		No. examined	No. with varices	No. examined	Avg P.P.,* cm H ₂ O	No. with P.P. >10 cm H ₂ O	No. with varices
Varices not present	0	21	0	9	5.0 ± .7†	0	0
	6	19	0	9	6.3 ± .4	0	0
	8	20	0	10	9.4 ± .4	5	0
Total		60	0	28		5	0
Varices present	10	20	11	10	10.3 ± 1.1	5	4
	12	19	7	9	11.9 ± 1.2	7	3
	14	19	7	10	10.4 ± 1.0	4	3
	16	18	8	8	9.4 ± .8	3	3
Total		76	33(43%)	37		19(51%)	13

* Portal pressure.

† Stand. error.

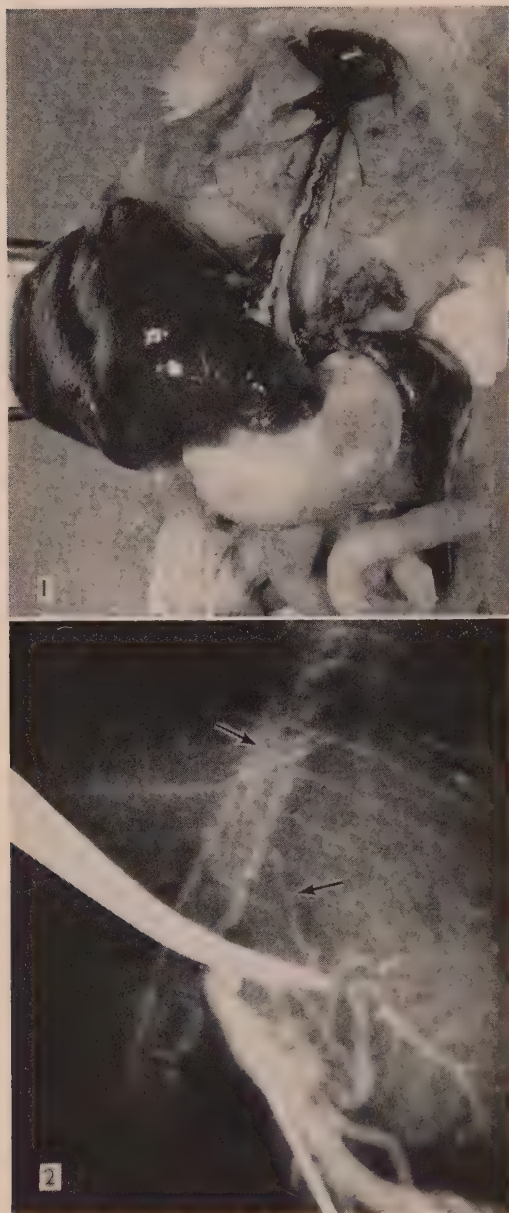


FIG. 1. 24 g mouse infected 14 wk—Yellow latex inj. of portal circulation. Note: hepatomegaly, splenomegaly, tortuous anterior and posterior periesophageal varices.

FIG. 2. 23 g mouse infected 14 wk. X-ray portogram—50% Hypaque inj. into portal vein with distal portion of vein clamped. Note: dilatation of portal vessels, tortuous esophageal varix (arrows).

compared to an average of 13.3 cm (range 8-18.5) in 13 mice with varices. Portal hypertension was considered to be present when

pressure rose above 10 cm of water. This occurred in 51% of the infected mice examined from tenth week of infection on.

Discussion. *S. mansoni* infection not only produces a significant rise in portal pressure in mice, but also causes development of esophageal varices. This provides a relatively rapid method for reproducing portal hypertension in the small laboratory animal. Studies are being planned in larger animals, keeping one particular limitation in mind, that of finding a good host for the particular species of schistosome utilized.

Many different methods (surgical, toxic and dietary) have been used in the past in unsuccessful attempts to produce consistent portal hypertension in experimental animals (1,3). One method frequently attempted has been to reproduce Laennec's cirrhosis, by either dietary or toxic means. It is of interest therefore that portal hypertension has been reported to occur in only 30% of human cases of Laennec's cirrhosis (3,5) while it has been estimated to be present in 40-70% of those with hepato-splenic schistosomiasis (5,6).

Another approach frequently employed in attempts to produce portal hypertension has been the introduction into the portal circulation of relatively inert substances of different particle sizes to create a mechanical block (1). The production of eggs in large quantities by the parasite simulates this method, but the eggs have one further unique ability. They excite a localized fibrotic reaction which effectively obliterates the entire portal area (7). This may be the final factor for the development of portal hypertension in mice infected with *Schistosoma mansoni*.

Summary. A useful technic for producing portal hypertension and esophageal varices in the laboratory animal has been described. Ten weeks following infection of mice with *S. mansoni*, there was a significant increase in portal pressure in 51% of our animals, and the development of esophageal varices in 43%.

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Geographic Variation in Plasma Protein Patterns of Snakes. (23953)

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We have reported(1) that electrophoretic patterns of plasma proteins of a number of species of reptiles and amphibians show distinct geographic variation. Some of these protein differences occur between currently recognized geographic races, whereas other differences appear to be independent of morphological variation. To our knowledge only 3 other examples of intraspecific variations in the pattern of plasma proteins have been reported. Deutsch and McShan(2) found distinct differences between patterns of the channel catfish and the white catfish which were considered at that time to be of the same species. Later studies, however, suggest that these two forms should be classified as different species(3). Smithies(4) demonstrated the presence of genetically determined differences between patterns of Caucasian and African races of man. Recently, Zweig and Crenshaw(5) have shown that 2 races of the turtle, *Pseudemys floridana*, can be differentiated by their plasma patterns. The present paper describes the nature of geographic variations found in plasma patterns of several species of snakes (common kingsnakes, racers, common water snakes, and common garter snakes).

Materials and methods. Snakes were either collected personally or obtained from naturalists in other sections of the United States. Locality of capture and scientific and common names(6) were recorded as exactly as possible for each animal. After a fast of one week, specimens were anesthetized with ether and bled from a cardiac puncture. The condition of the primary and secondary sex or-

gans was noted; animals were fixed in formalin, catalogued, and saved for deposit in the La. State Univ. Museum of Zoology. Blood was collected in heparinized tubes and centrifuged at 4°C. Plasma was separated immediately from cells and an aliquot of plasma was subjected to paper-electrophoresis on the same day that the animals were bled. In each electrophoretic run, a sample of human plasma served as a standard reference. Two-hundredths ml of plasma was applied across a 1 inch strip of Munckells 20 S filter paper and fractionated in barbital buffer (pH 8.6, ionic strength 0.05, 15% glycerol v/v) for 17 hrs at 170 v with an LKB paper-electrophoresis unit operating at 26 to 28°C. Details of the electrophoretic procedure have been published(1). After electrophoresis, each strip was dried and stained with bromphenol blue. The stained strips were made translucent with mineral oil and scanned at 590 m μ with Beckman DU spectrophotometer. Optical densities were plotted against distance along the strip, and the area under the resulting curves was used to calculate percentages of protein fractions. In order to eliminate possible variabilities due to the apparatus, samples of all populations being compared were rerun simultaneously on parallel strips. Fresh samples were not always available on the same day and frozen plasma (stored at -20°C), which produced patterns nearly identical to the fresh, was substituted.

Results. Plasma protein patterns of snakes characteristically exhibit 2 distinct groups of anodal fractions which are separated by an

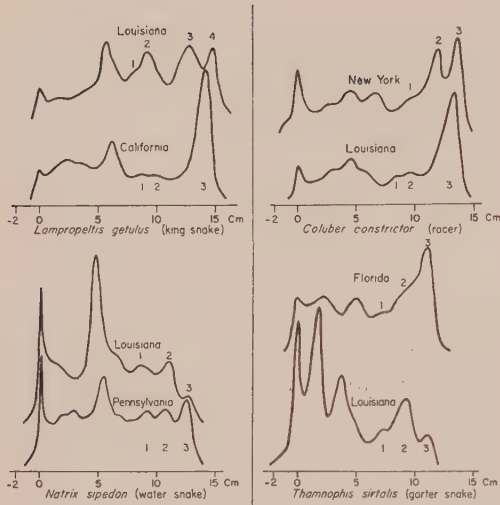


FIG. 1. Paper-electrophoretic patterns of plasma proteins of geographic variants of 4 species of snakes. Plasma was applied at 0 cm; anode is to the right. In pattern of La. specimen of *T. sirtalis* peak at 2 cm is caused by a protein fraction which appears in plasma of snakes during estrus. Only the numbered peaks (Fast Group fractions) are used in geographical comparisons.

area of low protein concentration (Fig. 1). The slower migrating proteins (Slow Group) exhibit one or two well resolved bands between 3 and 7 cm from starting line. The fast migrating proteins (Fast Group) consist of 3 or 4 bands between 7 and 15 cm from the starting line.

The pattern of Slow Group proteins is highly variable even in specimens from a single population. The quantity of these proteins increases with age. They contribute only a small percentage to the total plasma protein of young animals(7). In females of viviparous species such as *Natrix* and *Thamnophis*, not only does the total plasma protein increase at height of estrus, but a new fraction, which may include as much as 20% of total plasma protein, appears in the Slow Group(8). Such a fraction can be seen at the 2 cm position in the pattern of the Louisiana specimen of *T. sirtalis* (Fig. 1). Hemoglobin in plasma of hemolyzed samples migrates with Slow Group proteins(9). In contrast to the variability of the Slow Group, patterns of Fast Group proteins are relatively uniform in specimens from a single population. They are much less affected by such factors as age, sex and hemolysis. For these

reasons we believe that Fast Group proteins are more useful than Slow Group proteins in studies of geographic variation. In the comparisons of populations (Table I) we avoided using the Slow Group proteins by computing the percentage of Fast Group fractions relative to total Fast Group protein only.

A striking example of geographic variation in plasma protein is found in the patterns of the common kingsnake, *Lampropeltis getulus* (Table I, Fig. 1). A single protein band as the lead fraction is characteristic of Calif. specimens, whereas in kingsnakes from the southeastern United States the protein in the same region of the strip resolves into two bands. Plasma patterns of the two well known color variants of the California kingsnake are identical. Only minor differences were noted between patterns of kingsnakes from Ga., Ark., La., and Miss. The pattern of the blue racer, *Coluber constrictor*, from New York is similarly characterized by two well resolved lead fractions, whereas only one fraction is present in the same region of the strip in patterns of other racers sampled (Table I, Fig. 1).

Plasma patterns of common water snakes, *Natrix sipedon*, captured in different areas often exhibit quantitative differences in Fast Group fractions of similar mobility. Fast Group proteins of this species characteristically resolve into 3 fractions. In the patterns of the La. and Pa. specimens (Fig. 1) these fractions appear on the anodal side of the trough at 7.5 cm. In the lower Mississippi Valley, the region in which the races *pleuralis*, *fasciatus*, *confluens* and *clarki* (?) intergrade, populations living in close proximity often exhibit different patterns (Table I). For example, the pattern of a population from Lutchter, La., differs from that of a population from Westwego, La., which is only 30 miles distant and on the opposite bank of the Mississippi River. The pattern of the specimen from Bay St. Louis, Miss., is markedly different from that of the animal from Wesson, Miss., 150 miles to the north. The pattern of the Gulf marsh snake, *N. s. clarki*, differs in fraction mobility and other characteristics from *N. s. confluens* found in an adjacent but less saline environment.

TABLE I. Relative Migrations of Fast Group Fractions and Their Percentage of Total Fast Group Proteins.

Area of capture	No. of animals	Subspecies	Relative migration*†				% of fast group proteins†			
			1	2	3	4	1	2	3	4
<i>Lampropeltis getulus</i>										
Calif.	3	<i>californiae</i>	67 ± 2	77 ± 2	104 ± 3		12 ± 1	14 ± 2	74 ± 3	
Ga.	4	<i>getulus</i>								
Ark.	2	<i>holbrooki</i>	59 ± 3	70 ± 2	95 ± 4	112 ± 4	17 ± 4	24 ± 4	49 ± 6	20 ± 6
L.a.	21	"								
Miss.	2	"								
<i>Coluber constrictor</i>										
N. Y.	1	<i>constrictor</i>	69	88	102		25	37	38	
Fla.	1	<i>priapus</i>								
Miss.	2	"	57 ± 4	71 ± 3	95 ± 4		12 ± 4	21 ± 7	67 ± 8	
L.a.	14	<i>flaviventris</i>								
Ark.	2	"								
Calif.	2	<i>mormon</i>	61 ± 1	70 ± 3	96 ± 1		14 ± 3	5 ± 1	81 ± 3	
<i>Natrix sipedon</i>										
Pa.	1	<i>sipedon</i>								
N. Y.	2	"	64 ± 3	76 ± 4	87 ± 3		37 ± 6	28 ± 3	35 ± 4	
Mass.	2	"								
Ill.	2	<i>sipedon</i>	64 ± 2	75 ± 3	85 ± 1	93 ± 2	25 ± 2	25 ± 3	21 ± 3	29 ± 2
Mo.	2	<i>pleuralis</i>	66 ± 4	80 ± 2	91 ± 3		34 ± 3	18 ± 4	49 ± 2	
Miss.										
Wesson	1	<i>pleuralis</i> (?)	65	75	90		38	17	46	
Bay St. Louis	1	<i>confluens</i> (?)	65	80	93		55	21	24	
L.a.										
Lutcher	6	<i>confluens</i>	68 ± 2	85 ± 4	96 ± 3		41 ± 9	47 ± 8	12 ± 3	
Westwego	9	<i>confluens</i>	67 ± 3	81 ± 3	94 ± 4		54 ± 5	37 ± 8	9 ± 5	
Other areas	9	<i>confluens</i>	68 ± 4	83 ± 4	95 ± 4		50 ± 5	39 ± 6	11 ± 5	
Theriot	4	<i>clarki</i>	70 ± 3	80 ± 3	90 ± 2		30 ± 4	35 ± 2	35 ± 3	
Fla.	7	<i>pictiventris</i>	64 ± 4	79 ± 4	91 ± 4		31 ± 3	21 ± 3	48 ± 3	
<i>Thamnophis sirtalis</i>										
L.a.	4	<i>sirtalis</i>	67 ± 6	85 ± 5	102 ± 3		29 ± 2	54 ± 3	17 ± 2	
Minn.	6	<i>sirtalis</i>	65 ± 4	85 ± 3	98 ± 2		25 ± 3	39 ± 3	36 ± 3	
Fla.	5	<i>sirtalis</i>	63 ± 3	84 ± 3	97 ± 3		24 ± 4	33 ± 5	43 ± 2	
N. Y.	2	<i>sirtalis</i>								
Mass.	10	"	69 ± 4	84 ± 4	97 ± 4		30 ± 7	37 ± 7	34 ± 9	
Ore.	4	<i>concinus</i>								
Wash.	5	"	68 ± 4	80 ± 5	95 ± 3		26 ± 6	38 ± 5	36 ± 5	
Calif.										
San Joaquin	6	<i>fitchi</i>	74 ± 4	95 ± 5			25 ± 4	75 ± 5		
San Francisco	6	<i>tetrataenia</i>	70 ± 5	93 ± 4			22 ± 4	78 ± 5		

* Distance which fraction migrates $\times 100/\text{distance which reference human plasma albumin migrates (avg of } 12.7 \pm .8 \text{ cm)}$.

† Mean $\pm$ stand. dev.

The Fast Group proteins of the common garter snake, *Thamnophis sirtalis*, also generally resolve into 3 fractions. In the pattern of the eastern garter snake, *T. s. sirtalis* (Fig. 1), the trough which separates Slow from Fast Group proteins appears at 6 cm. Although La. and Fla. populations are currently recognized as members of the same subspecies, their patterns differ both in rate of migration and in relative amounts of Fast Group fractions. Other regional differences occur within this subspecies (Table I). Of the west coast

garter snakes, the pattern of *T. s. concinus* from Ore. and Wash. appears more similar to those of the eastern subspecies than to those of two Calif. races, *T. s. fitchi* or *T. s. tetrataenia*. Patterns of *fitchi* and *tetrataenia* differ from other subspecies of *T. sirtalis* but are quite similar to each other.

Discussion. Previous studies of geographic variation have dealt primarily with morphological differences between populations of a species which replace each other geographically (allopatric populations). More recent

studies reveal that physiological differences between such populations are prevalent(10) although less obvious. Our data show that modifications of plasma proteins are also present. These modifications are revealed by variations in position, number and quantity of protein fractions in paper-electrophoretic patterns of plasma.

Individuals of populations from a limited geographic area tend to have very similar plasma protein patterns, whereas those from widely separated areas usually have different patterns. Kingsnakes and racers are good examples of species in which geographic differences are reflected as variations in the position and number of fractions. In these species the lead protein migrates as a single peak in some populations but as two peaks of different mobility in other populations.

Quantitative variations in fractions of similar mobilities are well illustrated by a comparison of patterns of different populations of water snakes. In *Natrix sipedon*, for example, populations within the subspecies *N. s. confluens* have small, but probably significant, differences in the relative quantity of certain fractions. Even greater differences occur between characteristic subspecies (*N. s. confluens*, *N. s. sipedon* and *N. s. pictiventris*). Dissimilarities between the pattern of *N. s. clarki* and those of other races of *N. sipedon* are of sufficient magnitude to raise a question as to the justification of its being classified as a race of this species. Further sampling of the species and a better understanding of the significance of the patterns are necessary, however, before the technic of paper-electrophoresis can be utilized as a valid basis for taxonomic decisions.

It would be premature to assert that most geographic differences in plasma proteins are genetically determined. Several observations, however, favor this hypothesis: patterns of Fast Group proteins show little seasonal variation, only minor differences with age and insignificant differences between the sexes. Further, a Fast Group protein pattern may be either distributed uniformly over a large area or may be quite variable within a small area. Since electrophoretic patterns thus display the same type of variation and distribution as

morphological and physiological characteristics, it seems logical to propose that they are also inheritable and subject to natural selection.

In keeping with the above hypothesis is the interesting parallelism which appeared when patterns of *T. sirtalis* and *N. sipedon* from the delta region of S.E. La. were compared with patterns of their respective counterparts from N.E. states and Fla. In both species the concentration of the fastest moving fraction was much less in the patterns of La. samples than in those of the eastern populations. Further investigation revealed a similar relative fraction difference between La. and Fla. specimens of both *T. sauritus* (ribbon snake) and *N. cyclopion* (green water snake). In view of the fact that these four species are closely related (subfamily Natricinae) it seems plausible to suggest that due to a basically similar genetic potential they have utilized a common means of adapting to the delta environment.

We propose that in adapting to a local environment populations have accumulated genetic mutations which produce minor alterations of the plasma proteins, some of which affect their electric charge. These result in small alterations in electrophoretic mobilities or in the quantities of characteristic electrophoretic fractions. The amount of variation possible appears to be held within certain limits determined by a basic species pattern and consequently closely related species may show similar adaptations.

Summary. Distinct geographic differences between plasma proteins are revealed in kingsnakes, racers, water snakes and garter snakes by variations in the position, number and quantity of fractions of paper-electrophoretic patterns. The faster migrating fractions of the patterns are most useful in such comparisons as slower migrating fractions exhibit much physiologic variation.

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Typing of ECHO Viruses by a Complement Fixation Technic. (23954)

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Typing of ECHO viruses(1) by standard neutralization tests is an inconvenient and laborious procedure because of large number of prototypes. Recent findings, that complement-fixing (CF) antigens for ECHO viruses could be prepared consistently from monkey kidney tissue cultures and that these antigens reacted type specifically with ECHO antisera prepared in guinea pigs and monkeys(2), suggested the possibility of typing ECHO virus isolates by means of a CF technic. This preliminary report describes simple methods which have been successful in typing 28 ECHO virus isolates by such a procedure.[†]

Methods. CF antigens were prepared by inoculating each of 4 rhesus kidney tissue culture tubes containing 1 ml of the following: lactalbumin hydrolysate 0.5%, calf serum 2%, Earle's solution 97.5%, with 0.1 ml of undiluted fluid from the original or an early monkey kidney passage of the ECHO isolate. After complete degeneration of tissue had taken place, the tubes were frozen and thawed, and pooled tissue culture fluid from these tubes was used as antigen without centrifugation. Of 28 ECHO virus isolates tested, 15 were from a Washington, D.C. welfare institution(3), and 13 were from other

sources in Washington, D.C., and from other localities in U.S. and England (ECHO type 9).[‡] All were typed originally by neutralization tests except the ECHO type 10 isolates. The latter, however, produced the unique cytopathogenic effect characteristic of the prototype strain of ECHO 10 virus. The CF typing tests for most strains were done without prior knowledge of the neutralization test data. ECHO antisera used in CF tests had been prepared in monkeys(4).[§] Homotypic and heterotypic CF titers of these sera against CF antigens of ECHO prototype strains prepared in monkey kidney tissue culture are published elsewhere(2). Dilutions of monkey sera are shown in Table I and in each instance represented at least 4 units of antibody. These dilutions were determined by the desirability of conserving serum and the fact that 4 units of antibody have been used successfully for many years in this and other laboratories as the best working dilutions for purposes of titrating viral and rickettsial antigens. Complement fixation tests were performed by standard technic in this laboratory(2).

Results. Representative results of CF typing tests are shown in Table I. Each ECHO antigen reacted only with homologous antiserum, except the ECHO 8 antigens, which reacted with both type 1 and 8 antisera, and to

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‡ The authors are indebted to Dr. Karl Habel for supplying these 13 ECHO isolates.

§ The sera were obtained from Nat. Foundation for Infantile Paralysis.

TABLE I. Representative Results of ECHO CF Typing Tests. 4 = no hemolysis; 3, 2, 1 = different degrees of hemolysis; 0 = complete hemolysis.

Type of isolate by neutralization test	Type and reciprocal of dilution of ECHO antisera													
	E-1 256	E-2 128	E-3 128	E-4 128	E-5 256	E-6 256	E-7 256	E-8 256	E-9 128	E-10 128	E-11 128	E-12 128	E-13 256	E-14 256
ECHO type 3	0	0	4	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	4	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	3	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	2	0	0	0	0	0	0	0
8	4	0	0	0	0	0	0	4	0	0	0	0	1	0
9	0	0	0	0	0	0	0	0	4	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	4	0	0	0	0
11	0	0	0	0	0	0	0	0	0	4	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	3	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	4

a lesser extent with type 13. This, of course, was not unexpected in view of other data on the relationship of ECHO types 1, 8 and 13 (2, 4). The positive reactions were 4+ fixation in the majority of cases. In those few cases where the positive reactions were only 2+ or 3+ fixation, they were clearly separable from completely negative reactions. Polio-myelitis and Coxsackie B isolates (from the same sources as the ECHO isolates) which reacted in CF tests with homologous antisera gave no reaction with any of ECHO antisera.

Table II summarizes results of ECHO CF typing experiments with 28 ECHO isolates.

TABLE II. Number and Type of ECHO Isolates Typed by CF Technic.

ECHO type	No. of isolates	No. of antigens prepared from isolates	No. of antigens typed by CF technics
3	1	1	1
4	1	2	2
6	4	5	4
7	7	9	9
8	5	5	5
9	1	1	1
10	3	5	5
11	1	1	1
12	2	5	5
14	3	3	3
Total	28	37	36

Two or 3 antigens were prepared from some of these isolates and hence the total number of antigens tested was 37. All 28 isolates were successfully typed by this CF technic. One antigen from an ECHO type 6 isolate, prepared by inoculating monkey kidney tubes with undiluted material, and which caused complete degeneration of tissue in less than 24 hours, did not give any reaction. However, another antigen from the same isolate, prepared by diluting original tissue culture material 1:10,000 before inoculation of monkey kidney tubes, gave 4+ fixation with ECHO type 6 antiserum. No antigens were anticomplementary to the extent that they could not be typed.

These preliminary results indicate that the complement fixation test may be a faster and less expensive technic for typing of ECHO viruses than is the neutralization test.

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Chemotherapeutic Effectiveness of 1,1'-Trimethylene Bis (4-Formylpyridinium Bromide) Dioxime (TMB-4) in Experimental Anticholinesterase Poisoning. (23955)

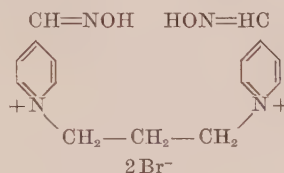
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Treatment of serious poisoning by phosphate and phosphonate esters of the "nerve gas" and insecticide type relies principally on prevention and control of the actions of acetylcholine(1,2,3). Long-lasting "irreversible" inhibition of body cholinesterase by these esters in small amounts is believed to account for their great toxicity(4,5) and control of their actions has centered on liberal use of atropine, long known for its capacity to block the actions of acetylcholine in many of its loci of action, including the central nervous system(1,2,6,7). Although the symptom-relieving and life-saving capacity of atropine is considerable when given in large amounts early in poisoning coupled with artificial respiration(8), its use is nevertheless limited. No other compounds with atropine-like properties, offering substantial improvement, have been found(8).

Repair of the biochemical lesion produced by nerve gas and similarly-acting phosphate esters (*e.g.* certain insecticides) has been a more recent approach to the problem(5). Restoration of cholinesterase activity to physiologically adequate levels with sufficient rapidity to avert or halt progress of poisoning serious enough to be beyond the control of atropine has been the object of this approach. The approach attempts to "cure the disease" by restoring the body mechanisms for dealing with its acetylcholine competently, rather than by treating symptomatically with a drug such as atropine. Suggestions that such an approach might be feasible appeared when it was demonstrated that certain oximes and hydroxamic acids may catalyze breakdown of such phosphate esters to innocuous products, may restore cholinesterase activity, or perform both functions *in vitro*(5,10). Limited *in vivo* data appear to demonstrate chemotherapeutic effectiveness of 2-pyridine aldoxime

methiodide (2-PAM) and other oximes against some phosphate esters(10,11). However, other investigators pointed out that in the absence of atropine the effectiveness of 2-PAM was negligible against phosphate esters(12). The present preliminary report deals with the effectiveness of a quaternized bispyridine aldoxime, one of a series emerging from a systematic synthesis of related compounds(13). This compound is 1,1'-Trimethylene bis (4-formylpyridinium bromide) dioxime (hereafter designated TMB-4) represented by the structural formula:



It reactivates GB-inhibited cholinesterases *in vitro* at a higher rate than does 2-PAM(13).

Methods. Briefly, the method for assessing chemotherapeutic effectiveness is as follows. Tests were conducted in 5 different species. A minimal number of animals was used in this routine screening method for preliminary evaluation of candidate agents. The toxic phosphate ester against which TMB-4 was tested was isopropyl methylphosphonofluoridate (GB). Two general types of tests were conducted: a) "prophylactic," in which TMB-4 was given not earlier than 2 minutes before GB to minimize excretion or other disposition of the compound; and b) "therapeutic," in which GB was followed not later than 30 seconds by TMB-4. Both compounds were in most cases given intravenously to avoid absorption problems; exceptions will be noted later. 2-PAM and TMB-4 were given routinely in maximum symptom-free doses, except in rabbits where equal amounts were used. GB was injected in appropriate multiples of its LD₅₀'s. Since it became apparent

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TABLE I. Protective Action of 2-Pyridine Aldoxime Methiodide (2-PAM) and of 1,1' Trimethylene Bis (4-Formylpyridinium Bromide) Dioxime (TMB-4) against Experimental "GB" Poisoning in Different Species.* *Poisoning dose of GB used: Mice—173 mg/kg (LD₅₀); rats—126 mg/kg (2 LD₅₀); rabbits—intrav. .340 mg/kg (20 LD₅₀), subcut. .700 mg/kg (20 LD₅₀); dogs and cats—intrav. .440 mg/kg (20 LD₅₀), subcut. .900 mg/kg (20 LD₅₀).*

	2-PAM					TMB-4				
	Survival ratio					Survival ratio				
	"Prophylactic" "Therapeutic"					"Prophylactic" "Therapeutic"				
	Dose, mg/kg	Alone	With atropine	Alone	With atropine	Dose, mg/kg	Alone	With atropine	Alone	With atropine
Mice	40	0/10		0/10		12	0/10		1/10	
Rats	40	0/6		0/6	1/6	25	0/6		1/6	6/6
Rabbits	5		0/5		2/6	5		2/5		4/6
Cats	40		2/5		5/5	20		5/5		3/5
Dogs	40		5/5		4/5	20		5/5		4/5

* For description of test, see text.

from its structure that TMB-4, like 2-PAM has little protective action in the absence of atropine(7), the latter was included, as indicated, in standard dose of 4 mg/kg in rats, 2 mg/kg in rabbits, and 0.5 mg/kg in cats and dogs. Use of the terms "prophylactic" and "therapeutic" is not intended to place interpretation on mechanism of action, but rather refers to temporal relation of administration of toxic and treatment agents to test animals. Under conditions of our tests, 2-pyridine aldoxime (2-PAM) exerts insignificant prophylactic and therapeutic action in mice and rats.

Atropine, which is known to be relatively ineffective against anticholinesterase poisoning in rodents, improved both actions of 2-PAM somewhat; the most striking contribution of atropine is seen in cats and dogs. Whereas rabbits, cats, and dogs die of intravenous lethal dose of GB within a few minutes, a combination of 2-PAM and atropine protects these animals considerably against 20 LD₅₀'s of GB prophylactically and therapeutically in doses which are ineffective by themselves. In rats TMB-4 is more effective than 2-PAM. However, recovery time from symptoms of

TABLE II. Recovery Periods of Animals Poisoned with GB and Treated with 2-PAM or TMB-4 as Adjuncts to Atropine.

Species	2-PAM				TMB-4			
	"Prophylactic"		"Therapeutic"		"Prophylactic"		"Therapeutic"	
	No. of animals tested	Remarks	No. of animals tested	Remarks	No. of animals tested	Remarks	No. of animals tested	Remarks
Rats			6	5 died within 10 min. 1 recov. within 60 min.			6	Recov. within 15 min.
Rabbits	5	Died within 3 min.	6	4 died within 10 min. 2 recov. within 3 hr	5	3 died within 10 min. 2 recov. within 30 min.	6	2 died within 20 min. 4 recov. within 2 hr
Cats	5	3 died within 15 min. 2 recov. within 5 hr	5	Recov. within 5 hr	5	Recov. within 5 hr	5	2 died within 24 hr 3 did not recover within 24 hr
Dogs	5	Did not recov. within 24 hr	5	1 died within 60 min. 4 did not recover, 24 hr	5	Recov. within 1½ hr	5	1 died within 60 min. 4 recov. within 3 hr

poisoning was considerably shorter in all species except in cats when TMB-4 was administered with atropine and used as "therapeutic" agent. It is to be noted that in these "therapeutic" tests, GB was given subcutaneously only because death from 20 intravenous LD₅₀'s of GB is so rapid that timely administration of treatment agent is practically impossible. The intravenous treatment was started as soon as poisoning symptoms were visible. Effectiveness of TMB-4 in the different species may be compared with that of 2-PAM by reference to Tables I and II.

Discussion. Conditions of "prophylactic" and "therapeutic" tests allow minimal protective action to appear, while at the same time requiring antidotal action significantly above that of atropine to be detected. The intravenous route of administration avoids most difficulties in interpretation arising from changes in rate and extent of absorption of toxic and treatment compounds. The use of atropine recognizes the necessity for blocking immediate and very rapid lethal action of GB, by blocking of muscarinic and central nervous system action of acetylcholine(7). This provides more time than otherwise possible for the action of cholinesterase restoratives such as TMB-4, as revealed by *in vitro* studies(13), presumably to bring tissue cholinesterase to lifesaving or even physiologically adequate levels. The latter deserves emphasis, for survivors among cats and dogs exposed to sublethal doses of GB and treated with atropine alone show weakness, gait abnormalities and other signs of incomplete recovery for many hours or days, whereas in rabbits, cats and dogs fast recovery was found when treated with TMB-4 and atropine prophylactically. Similarly rats, rabbits and dogs exposed to multiple LD₅₀'s of GB show practically complete recovery in a few hours when treated also with TMB-4. Further study is obviously required to correlate rates of tissue cholinesterase restoration with return of function to levels required to ensure survival and normal activity. In most of these tests a lower dose of TMB-4 was administered than of 2-PAM for the reason given above; however, this does not necessarily indicate a lower therapeutic index of the latter compound. In this

preliminary study the number of animals is too small for statistical evaluation of the superiority of one compound above the other. However, comparison of effectiveness of these compounds suggests that TMB-4 is at least equivalent if not superior to 2-PAM in its use as therapeutic agent in experimental anticholinesterase poisoning.

The quaternized bispyridine aldoxime with a trimethylene bridge between pyridine aldoxime portions of the molecule, TMB-4, is the most effective against experimental nerve gas poisoning and least toxic of the group. With increasing chain length the toxicity rises and therapeutic effectiveness diminishes.

The compound, 1, 1'-trimethylene bis (4-formylpyridinium bromide) dioxime (TMB-4), reported here seems very promising by comparison with 2-pyridine aldoxime methiodide (2-PAM), since when combined with atropine its effectiveness in immediate protection against lethal action and especially in symptomatic recovery from poisoning by GB is such as to merit trial against poisoning by other anticholinesterase agents such as parathion, tetraethylpyrophosphate, and diisopropyl phosphorofluoridate.

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Kinetics of Tyrosine Oxidation by Crude Tyrosinase Preparations from *Neurospora crassa*.* (23956)

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The ascomycete *Neurospora crassa*, strain 15300, is a mutant which differs from the wild-type at the albino-2 locus(1). Extracts of 15300 which contain the enzyme tyrosinase, are capable of oxidizing tyrosine, dopa (3-4 dihydroxyphenylalanine), or other mono- or dihydroxyphenols to corresponding 3-4 quinones, which are eventually converted to colored products (dopachrome in the case of tyrosine and dopa). We have for some time been engaged in an analysis of genetic control of synthesis and specificity of tyrosinase in strain 15300(2,3,4). The necessity to examine a large number of genetically different cultures has dictated the use of crude enzyme preparations and spectrophotometric methods. This paper presents a study justifying the use of such methods and the use of crude extracts as a source of enzyme for determination of enzyme activity and specificity as reliable and dependable and not different from that of purified enzyme preparations.

Materials and methods. Large quantities of mycelium, from a single ascospore culture (15300-131a) isolated from the cross of 15300 A x 15300a, were obtained by growth on liquid minimal medium for 33 days at 25°C. After harvest, the mycelium was washed thoroughly and lyophilized. Enzyme preparations were made by homogenizing 23 mg of lyophilized mycelium in 2.50 ml of buffer (0.85% NaCl + 0.1 M phosphate pH 6.7) at 5°C for 5 minutes in an all-glass homogenizer. Aliquots of the supernate, collected after centrifugation at 5000 rpm for 20 minutes at 5°C, were tested for activity by meas-

uring the rate of dopachrome formation spectrophotometrically, *i.e.* at 305 m μ or 475 m μ in a Beckman DU spectrophotometer. Reaction mixtures consisted of 0.25 ml of supernate + 3.25 ml of buffer containing defined amounts of l-tyrosine as substrate. Further details are provided elsewhere(4).

Results. Kinetics of dopachrome formation. The course of dopachrome formation in our reaction mixtures follows closely the course of tyrosine oxidation demonstrated by other workers using manometric methods and purified enzyme preparations (Fig. 1). There is a distinct induction period, followed by period of linearity, ultimately falling off as the reaction proceeds. Furthermore, the rate of dopachrome formation during the linear period is related to substrate concentration as expected on the basis of the Lineweaver-Burk modification of the Michaelis-Menten relationship (Fig. 2). This makes it possible to calculate both V_{max} , the reaction rate expected when substrate is unlimited, which measures enzyme concentration under these circumstances, and the apparent Michaelis Constant (K_s) of the enzyme. The rate of dopachrome formation is influenced by pH (Fig. 3), falling symmetrically on either side of pH 7.4, which can be taken as the pH optimum of the enzyme. All these observations strongly suggest that the spectrophotometric method is satisfactory for the measurement of tyrosine oxidation by crude tyrosinase preparations from *Neurospora*. A direct demonstration was nevertheless deemed important, and studies of the relationship of tyrosine utilization to dopachrome formation in our reaction mixtures were therefore undertaken.

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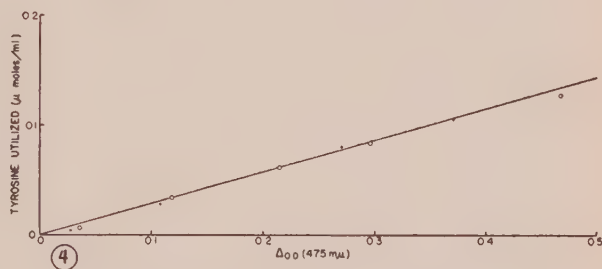
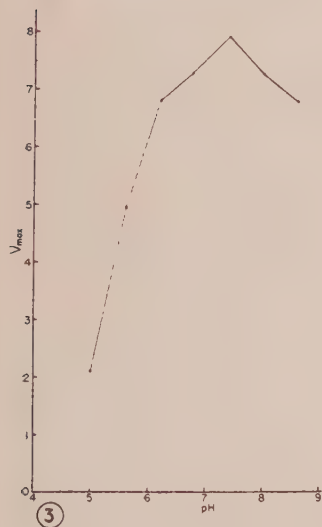
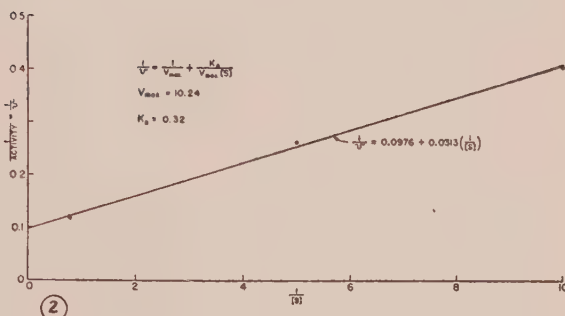
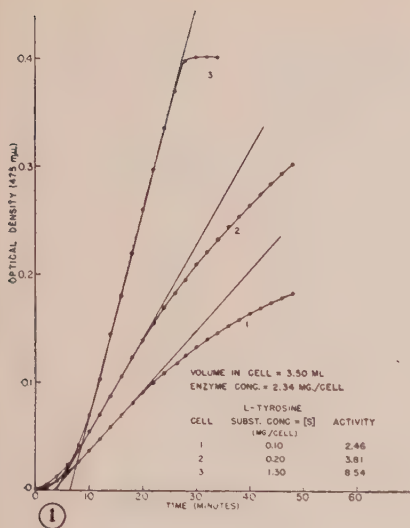


FIG. 1. Oxidation of tyrosine by extract of 15300-131a mycelium. Activities calculated from slopes of linear portions of respective curves = change of optical density/min./mg of lyophilized mycelium.

FIG. 2. Lineweaver-Burk kinetics of tyrosine oxidation by extract of 15300-131a mycelium. [S] = substrate conc. v = activity (see Fig. 1). v_{max} = reaction rate when substrate is unlimited. K_s = apparent Michaelis Constant.

FIG. 3. Effects of pH on tyrosinase activity of extract of 15300-131a mycelium. v_{max} calculated as in Fig. 2. Dopachrome production measured at 475 $m\mu$.

FIG. 4. Relationship between tyrosine utilized and change in optical density during incubation of reaction mixture containing extract of 15300-131a mycelium. Open and solid circles represent results of 2 separate experiments.

Relationship of tyrosine utilization to dopachrome formation. Our task was to determine whether each mole of tyrosine utilized in the reaction mixture gives rise to one mole of dopachrome. Concentration of the latter was determined spectrophotometrically. Concentration of tyrosine was determined by means

of a chromatographic method. Preliminary investigation showed that known quantities of tyrosine could be separated from amino acid, peptide, and protein mixtures by chromatography, detected on filter paper by complexing with ninhydrin, rendered light stable and methanol soluble by further complexing with

TABLE I. Relationship between Tyrosine Utilized and Dopachrome Produced by Reaction Mixtures Containing 15300-131a Extract.

Incubation time (min.)	Exp. 1			Exp. 2		
	Tyrosine utilized (μ moles/ml)		Dopachrome produced (μ moles/ml)	Tyrosine utilized (μ moles/ml)		Dopachrome produced (μ moles/ml)
	Observed	Calculated		Observed	Calculated	
0	0	0	0	0	0	0
5	.0036	.0078	.0078	.0069	.0104	.0104
10	.0285	.0313	.0312	.0345	.0342	.0341
15	.0755	.0560	.0558	.0620	.0621	.0618
20	.0810	.0783	.0780	.0841	.0858	.0855
25	.1060	.1073	.1069			
30				.1284	.1354	.1349

copper(5), and recovered quantitatively by elution from the paper with methanol. The optical density at 512 $m\mu$ of these methanol eluates is linearly related to amount of tyrosine initially deposited on the chromatographic paper in the range of 1.3 to 25.0 μ g, thus affording a convenient method for estimation of free tyrosine in a mixture. Reaction mixtures were prepared in sets of 6, containing equal amounts of enzyme extract and equal amounts of tyrosine. These were incubated and optical density measurements at 475 $m\mu$, measuring dopachrome concentration, were made at precisely 2 minute intervals. During the experiment, and at time intervals of 5, 10, 15, 20, 25, and 30 minutes, the reaction was arrested in a single cell by mixing its contents with an equal volume of absolute ethanol. At end of experiment, each mixture was subjected to chromatography and the amount of tyrosine remaining determined by the method described above. The amount of tyrosine utilized was then determined by difference.

Fig. 4 clearly demonstrates that the relationship between tyrosine utilized and increase of optical density at 475 $m\mu$ is a linear one. The relationship between tyrosine utilized in a reaction mixture and its increase in optical density, as determined by the least squares method, is tyrosine utilized (μ moles/ml) = 0.290 X optical density increase (475 $m\mu$). The linearity of this relationship suggests that there are no other pathways of tyrosine utilization in the reaction mixtures, and that there is an equimolar relationship between amount of tyrosine utilized and amount of dopachrome produced. This last conclusion is more directly demonstrated by calculating the

amount of dopachrome produced from the observed increase in optical density and the molecular extinction coefficient value of dopachrome as given by Mason(6). Table I compares such values with both the observed amount of tyrosine utilized and the amount of tyrosine utilized as calculated from the relationship presented in Fig. 4. Since there is no significant difference between amount of tyrosine utilized (observed or calculated) and amount of dopachrome produced, it may be concluded that an equimolar relationship exists between the 2, and that the use of crude extracts and spectrophotometric methods is justified.

Analysis of induction period. The induction period observed in oxidation of tyrosine by purified tyrosinase is generally attributed to the necessity for a catalytic amount of dihydroxyphenol for the reaction to proceed(7). Our crude extracts, however, contain protyrosinase which undergoes enzymatic activation during incubation, even in the absence of substrate(4). The induction period which we observe could be attributable to both an initial absence of required dihydroxyphenol, and to an actual increase in amount of enzyme during the reaction due to protyrosinase activation.

That this is indeed the case is demonstrated by the effects of incubation of enzyme extract at 5°C in the absence of substrate (Fig. 5). During the first 30 minutes of incubation enzyme activity (*i.e.*, slope of linear portion of curve) increases and the induction period is shortened. Other work(4) has demonstrated that most of the protyrosinase undergoes activation during this period. It is therefore

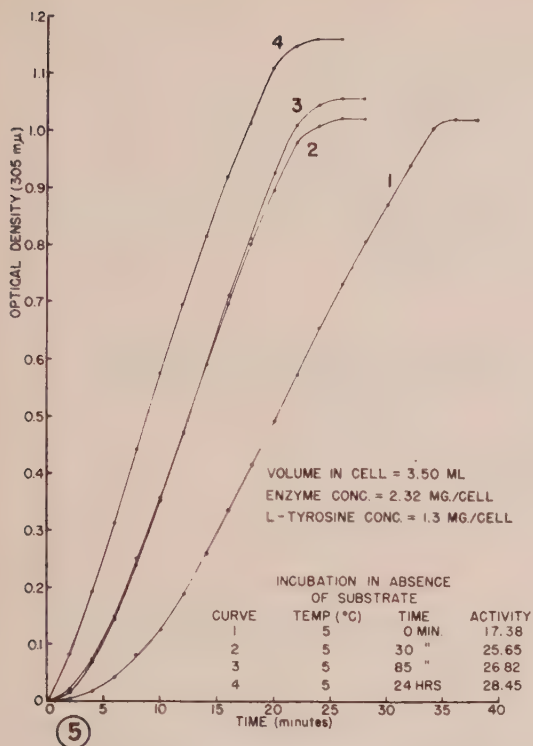
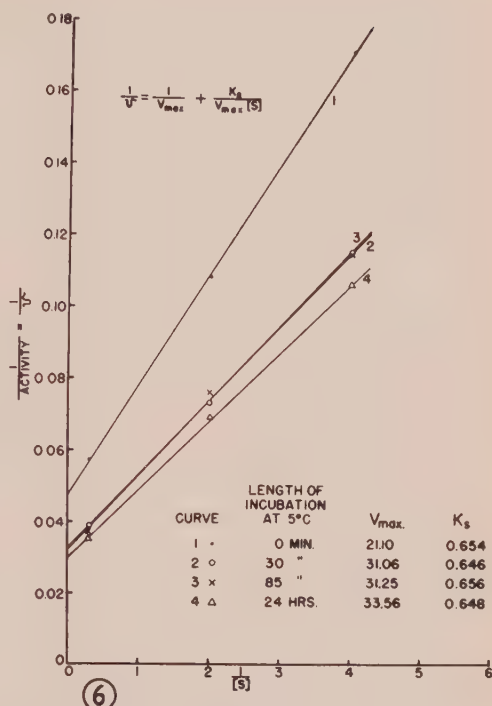


FIG. 5. Effects of incubation of 15300-131a extract in absence of substrate.

FIG. 6. Effects of incubation of 15300-131a extract in absence of substrate on Lineweaver-Burk kinetics of subsequent tyrosine oxidation.



not surprising to note that further incubation results in no further increase in enzyme activity, but does lead to eventual disappearance of the induction period.

These conclusions are better demonstrated when the data are subjected to Lineweaver-Burk treatment (Fig. 6). V_{max} , which under these conditions measures enzyme concentration, increases during the first 30 minutes of incubation, but undergoes no further change in spite of progressive shortening of the induction period. It is interesting to note that K_s does not change during incubation, thus confirming our previous conclusion(4) that the tyrosinase resulting from protyrosinase activation is the same as that originally present.

It may therefore be concluded that shortening of the induction period during the first 30 minutes of incubation is largely attributable to conversion of protyrosinase to tyrosinase, while the subsequent disappearance of the induction period is probably the result of the slow production of dopa or some other dihydroxyphenol from small amounts of precursors present in the extract.

When a fresh extract is tested for tyrosinase activity, both mechanisms will contribute to the observed period of induction.

Summary. 1. Oxidation of tyrosine by crude enzyme extracts of *Neurospora* mycelium, as measured by spectrophotometric determination of dopachrome formation, follows a course similar to that exhibited by purified tyrosinase. 2. The reaction exhibits conventional Michaelis-Menten kinetics with a pH optimum of 7.4. 3. Each mole of tyrosine utilized during the reaction is converted into a mole of dopachrome, thus demonstrating that no enzymatic systems utilizing tyrosine other than tyrosinase are present, and that the use of crude extracts and spectrophotometric methods is justified. 4. The induction period exhibited during the oxidation of tyrosine by the crude extracts results from both the production of catalytic amounts of dihydroxyphenols and conversion of protyrosinase to tyro-

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Antiaccelerator, Coronary Dilator and Certain Other Pharmacologic Actions of New Poly-Methoxyphenyl Derivatives. (23957)

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(Introduced by F. P. Luduena)

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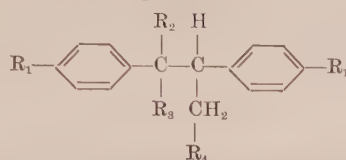
A specific new pharmacologic activity, the cardiac antiaccelerator effect, was described by Kraye and his associates (for review, cf. 1). This activity was found among naturally occurring veratramine and solanum alkaloids (1,2). More recently, a similar effect was described by Margolin and his associates (3) in the case of certain synthetic steroids with alkamine substitutions in position 16. It was conceived that relatively simple polyphenyl molecules with a lipophilic appendage may exhibit similar pharmacologic properties. This paper describes antiaccelerator potency data obtained in the course of screening of a series of such compounds as well as additional pharmacologic and toxicity results obtained with 2 particularly promising compounds of this series.

Methods. The blockade of positive chronotropic effect of epinephrine was tested in rabbit heart Langendorff preparations perfused at 25°C with oxygenated (95% O₂, 5% CO₂) Ringer-Locke by means of an apparatus (4) that permitted measurement of coronary flow. Cardiac rate was measured by means of Palmer drop recorder and amplitude of cardiac beat by means of a light isotonic lever. Epinephrine bitartrate was prepared in Ringer-Locke (5-10 µg/cc) with sodium bisulfite, final concentration 0.1%, acting as

antioxidant. Epinephrine was perfused at 1 cc/min. through a side arm provided with a flow meter. The compounds tested were solubilized in weak HCl and injected in 0.1 to 0.2 cc into the perfusion cannula near the heart. The reference compound, veratramine, was solubilized as described by Kraye (5); veratramine doses refer to the base. The positive chronotropic effect of epinephrine amounted in 20 tests to an average of 36 beats/minute and decreased by an average of 5 beats, or 14%, at end of 2 to 4 hours. In actual testing, inhibition of acceleration of 30% or more was considered significant. Anti-accelerator doses effective in 50% of hearts (AED₅₀) were determined from a dose-frequency curve according to the method of Wright (6); 3 to 5 doses were spaced at 0.5 logarithmic intervals, 3 to 5 hearts/dose. Additional measurements of the effect of compounds tested on coronary flow were carried out by the method of Luduena, Miller and Wilt (7). The acute LD₅₀ values and their standard error were measured in mice by the method of Miller and Tainter (8). Irritancy was determined in rabbits by means of trypan blue test (9,10). Local anesthetic potency was determined in guinea pigs by the intradermal wheal test (11). The cardiac rate was measured in atropinized dogs under chloralose or barbiturate anesthesia by means of a Palmer Recorder and a pressure transducer (12). The blockade by the com-

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TABLE I. Antiaccelerator and Coronary Dilator Action of Polyphenyl and Reference Compounds.



Compound No.	R ₁	R ₂	R ₃	R ₄	Antiaccelerator effect, AED ₅₀ , μg/heart	Coronary dilatation	
						Coronary flow increase, %*	% papaverine†
1	OCH ₃	—	4-methoxyphenyl	N Me ₂	31	125	230
2	"	OH	Isobutyl	"	48	105	115
3	"	"	Cyclo-hexyl	"	54	96	
4	"	"	Hexyl	"	50	70	
5	"	"	Isobutyl	1-piperidyl	23	62	≤50
6	"	"	Octyl	N Me ₂	≥100		
7†	H	"	Isobutyl	"	16.5	55	
8†	H	"	Phenyl	"	≥50	slight	
Veratramine					18	none	.2
Papaverine					≥200	89§	100

* Coronary flow was measured following epinephrine perfusion and inj. of compound tested. Avg increase was calculated at the AED₅₀ value.

† Technic of Luduena *et al.* (7). ‡ Known compound. § At 50 μg/heart. || Double bond at the R₂, R₁, carbon.

pounds tested of the epinephrine pressor and of acetylcholine vasodepressor responses, and of the response of nictitating membrane to faradic sympathetic stimulation was measured by conventional methods in dogs and in cats.

Results. The bio-comparison of antiaccelerator potency of the polyphenyl compounds and of veratramine was carried out on the epinephrine-driven isolated rabbit heart. Epinephrine infusion produced well-known inotropic, chronotropic and coronary dilator actions. The active polyphenyl derivatives reduced the positive chronotropic effects of epinephrine without affecting much its inotropic action, in which they resembled veratramine(1). The AED₅₀ values for cardiac slowing of all compounds tested, including papaverine and veratramine, are shown in Table I. Several new compounds approximated, in their activity, veratramine. The polyphenyl compounds differed unexpectedly from the veratramine in that several of them caused additional coronary dilatation (Table I), while veratramine produced none as already shown by Krayner(5). The dilator effect of compounds 1, 2 and 5 and of veratramine was tested also by a technic not involv-

ing epinephrine(7). There was a good qualitative agreement between the 2 sets of data (Table I). The coronary dilator effect of the new compounds seemed independent of the anti-accelerator action, since it was not parallel with the extent of the latter (Table I) and since, frequently, it obtained at doses which did not slow the heart. In particular, the effect of compound 1 was more than twice that of papaverine (Table I).

Autonomic action, particularly general sympatholytic activity could account for the blockade by the polyphenyl compounds of the chronotropic cardiac effect of epinephrine. Compounds 1 and 5 were found, however, to be incapable of blocking either the pressor effects of epinephrine in dogs or the withdrawal of the nictitating membrane on faradic stimulation of the cervical sympathetic in the cat (Table II); in this they resemble veratramine (5,13) and steroids studied by Margolin *et al.* (3). They were also found incapable of stimulating frog rectus and of cholinergic blockade in the dog (Table II). Another pharmacologic activity that could be involved in cardiac slowing is irritancy and/or local anesthetic action(11,14); both could be expected

TABLE II. Pharmacologic Properties of Compounds 3-Dimethyl-amine 1,1,2-tris-(4-methoxyphenyl)-1-propene and 2,3-bis (4-methoxyphenyl)-5-methyl-1-(1-piperidyl)-3-hexanol, and of Veratramine.

Test	Route	Compound		
		1	5	Veratramine
Toxicity in mice, LD ₅₀ , mg/kg	Intrav. Oral	30 ± 1 385 ± 85	31 ± 2.1	3.68 ± .24 13 ± 2.1
Approx. toxicity in unanesthetized dogs, mg/kg	Intrav. Oral	>10 >25	> 5 >20	.3 2.0
Irritancy, threshold conc., %		.25	.032	.10
Local anesthetic potency, % procaine		130	25	Convulsions at .2 mg/kg
Sympatholytic activity				
Reversal of epinephrine pressor effect in dogs, mg/kg	Intrav.	Slight blockade	None at 8	
Blockade of nictitating membrane response in cats, mg/kg	"	Slight at 4-10	<i>Idem</i>	
Anticholinergic action				
Blockade of acetylcholine depressor effect in dogs, mg/kg	"	None at 10	None at 10	

from polyphenyl compounds(11,14). Actual tests showed that compounds 1 and 5 exhibit local anesthetic potency (Table II). However, compound 1, almost as potent an anti-accelerator as compound 5, is many times weaker than the latter in its irritancy and local anesthetic action. In addition, procaine and a 2-alkoxy thiolbenzoate, a new compound with the anesthetic potency about 100 times that of procaine(15) were tested in the isolated heart for their possible antiacceleratory effects. Neither produced cardiac slowing short of complete AV block and ventricular fibrillation which occurred with about 3 mg per heart of procaine and 25 μ g per heart of thiolbenzoate.

It was of interest to test whether or not the antiaccelerator data obtained in the isolated heart would carry over into the intact animal. Compounds 1 and 5 exhibited antiaccelerator action in 15 atropinized and/or vagotomized dogs under pentobarbital or chloralose anesthesia in doses of from 0.3 to 0.5 mg/kg. Epinephrine infusion seemed not essential for this action which agrees with several experiments carried out without epinephrine on the isolated heart. There was little or no effect on mean arterial blood pressure whether in presence or in absence of epinephrine. Compounds 1 and 5, given i.v. in doses of from 0.5 to 2 mg/kg, produced also cardiac slowing in trained unanesthetized dogs (7 experiments).

In some instances, the rate decreased from 75 to 50 beats per minute and was maintained at the low level for 1 to 2 hours.

Sympatholytics produce usually compensatory tachycardia. A phenoethiazine sympatholytic(16) was used in trained unanesthetized dogs to produce a tachycardia of up to 200 beats per minute lasting for 5 hours or more. Compounds 1 and 5, 2 mg/kg i.v., or 5-10 mg/kg orally, blocked tachycardia and a rate of 60-70 per minute persisted for up to 4 hours. Electrocardiograms (Lead 2) of the trained unanesthetized animals indicated that the slowing of the rate, whether following experimental tachycardia or in untreated dogs, was a sinus bradycardia with little change in the QRS interval, *i.e.*, with little change in conduction.

Of particular interest in this series of experiments was that while compounds 1 and 5 showed no side actions at doses specified, veratramine produced in intact dogs considerable toxicity (Table II). In anesthetized atropinized dogs (17 experiments) veratramine slowed the heart at levels of 0.02-0.05 mg/kg and produced tremors and analeptic actions. In unanesthetized animals tachycardia, nausea, salivation and excitement appeared with doses of 30 to 40 μ g/kg, i.v., and tremors, convulsions and vomiting—with doses of 120-240 μ g/kg, i.v., or 2 mg/kg, orally. Two dogs were killed with 0.3 mg/kg, i.v. This indi-

cation that toxicity of veratramine is much higher than that of the polyphenyl compounds was borne out by the LD₅₀ data in mice (Table II).

Discussion. The prototype antiaccelerator agent, veratramine, exhibits 2 main pharmacologic actions: it blocks cardiac chronotropic effect of epinephrine, and initiates a complex reflex activity leading to hypotension and bradycardia ("Bezold effect," for review see Kraye and Acheson, 17, and Dawes and Comroe, 18). The present data suggest that the new polyphenyl compounds are antiaccelerators probably devoid of the hypotensive activity. Particularly, compounds 1 and 5 exhibit a specific antiaccelerator action independent of extracardiac sympatholytic and adrenolytic effect, not due to vagal slowing and not related to an effect on conduction which obtains with local anesthetics (*cf.* lack of antiaccelerator action of antiarrhythmic agents and local anesthetics, Kraye *et al.*, 19). Accordingly, the receptor of the "pacemaker tissue" (5) can be suggested as the specific site of antiaccelerator action of veratramine and certain related steroids, as well as of the present polyphenyl compounds. This receptor, although activated by epinephrine and norepinephrine differs in an interesting fashion from other sympathetic receptors in that it cannot be blocked by sympatholytic agents even when used in doses many times those sufficient to abolish vasoconstriction actions of epinephrine and of other sympathomimetics (20,21). Thus, sympatholytics cannot prevent tachycardia provoked by their own vasoconstrictor blockade and hypotension. On the other hand, the polyphenyl antiaccelerators are capable as shown at present, to differentially block the "primitive" (22) receptor of the pacemaker. To further characterize this receptor it should be said that all antiaccelerators described so far possess a relatively rigid polyring nucleus with a secondary or a tertiary N-containing ring, or with a nitrogen-bearing sidechain. Thus, they seem chemically related to endocrine agents; polyphenyl compounds described at present are diethylstilbestrol derivatives, while veratramine, solanum alkaloids and compounds described by Margolin *et al.* (3) are steroids re-

lated to testosterone.

Toxicity of veratramine and of steroids described by Margolin *et al.* limited their therapeutic application (3,23). Since the compounds studied at present are many times less toxic than veratramine (Table II) and since they have the additional advantage of being coronary dilators, their clinical trial has been initiated.

Summary. 1. A significant cardiac antiaccelerator activity was found among members of a new synthetic series of polymethoxyphenyl amines. 2. When tested on the isolated, epinephrine driven rabbit heart their potency was found in several instances to be comparable to that of veratramine. 3. They could produce also cardiac slowing in anesthetized and unanesthetized dogs as well as block, in unanesthetized dogs, compensatory tachycardia due to administration of a sympatholytic. 4. Some of the compounds were found to be coronary dilators, in one case 2-3 times more potent than papaverine. 5. Since the polyphenyl compounds were found not to be sympatholytic or adrenolytic, since their action is extra-vagal and independent of their local anesthetic activity, the possibility is suggested they have a specific site of action at the "primitive" pacemaker receptor.

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Influence of Adrenal Hormones on Lactic Acid Content of Rat Brain Tissue. (23958)

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An increase in lactic acid content of brain tissue in hypoxic animals has been established by various investigators(1-4). When brain tissue is analyzed one to 3 hours post mortem the lactic acid level may increase 4- to 5-fold (4). This probably is the result of endogenous enzymatic activity on lactic acid precursors such as glycogen, glucose or other carbohydrate material. There is considerable evidence to show that adrenal steroids are involved, in a permissive manner, in carbohydrate metabolism(5). This fact coupled with the almost exclusive use of carbohydrate in normal brain metabolism, suggested a study of the lactic acid content in brain tissue as influenced by hypoxia and adrenal hormones.

Methods. Male Sprague-Dawley rats weighing about 350 g were used. They were divided into 2 main groups; intact and adrenalectomized. All animals received Purina Laboratory Chow and water *ad libitum*, with the adrenalectomized animals receiving physiological saline in place of water. Four days post-operatively, the animals were placed into 2 subgroups; a ground level group which will be referred to as the "non-hypoxic" group and an altitude group which will be designated as the "hypoxic" group. Hypoxia was produced by taking the animals to a simulated

altitude of 30,000 feet (225.6 mm Hg) for a period of 105 minutes in a decompression chamber. This time and altitude were found to give a large increase in lactic acid content of brain tissue in intact control animals. At the end of experimental or control period, the animals were decapitated in their respective environments; *i.e.*, at ground level or at altitude. The decompression chamber was routinely returned to ground level within 5-6 minutes following decapitation. All heads were left intact at room temperature (23°-27°C) for a period of 3 hours, after which the brains were removed, blotted of excess blood, and homogenized in glass homogenizers with 10% trichloroacetic acid (10% homogenate). The lactic acid content of the whole brain was determined by the method as described by Van Fossan(4). Lactic acid content of brains from intact and adrenalectomized animals was determined under non-hypoxic and hypoxic conditions to obtain control data. In comparison, brain lactic acid content of adrenalectomized animals receiving the following adrenal hormones and dosages was determined: (a) epinephrine, 0.15 mg in sesame oil given subcutaneously 15 minutes prior to ascent to altitude; (b) corticosterone, 1.0 mg in saline given subcutaneously at 4 two-hour

TABLE I. Brain Lactic Acid of Intact and Adrenalectomized Rats.[†]

Treatment	N	Intact		Adrenalectomized		
		$\bar{X}$	S.E.	N	$\bar{X}$	S.E.
Non-hypoxic	10	115 $\pm$ 4		10	113 $\pm$ 5	
Hypoxic	10	216* $\pm$ 11		10	136 $\pm$ 12	
" + sesame oil				4	131 $\pm$ 3	
" + epinephrine	9	240* $\pm$ 10		9	217* $\pm$ 11	
" + saline				4	106 $\pm$ 17	
" + hydrocortisone	5	217* $\pm$ 10		9	220* $\pm$ 10	
" + corticosterone				9	243* $\pm$ 8	
" + cortisone				9	210* $\pm$ 18	
(Acclimatized to 18,000')						
Killed at ground level	5	124 $\pm$ 4				
" " 18,000'	5	137 $\pm$ 8				
" " 30,000'	8	171* $\pm$ 16				

* $P < .01$ in reference to non-hypoxic values.[†] mg of lactic acid/100 g of tissue.

intervals prior to exposure to altitude; (c) hydrocortisone, 0.33 mg in saline given as in b above; (d) cortisone, 0.5 mg in saline given as in b above. In addition, the brain lactic acid content of rats that had been maintained at a simulated altitude of 18,000 feet for a period of 12 weeks was also determined after killing at ground level, 18,000 and 30,000 feet.

Results. Brain lactic acid content of intact and adrenalectomized nonhypoxic animals was similar, 115 ± 4 and 113 ± 5 mg/100 g of brain tissue respectively. When similar groups were made hypoxic, the brain lactic acid content of adrenalectomized animals was considerably less than that of intact animals, 136 ± 12 and 216 ± 11 mg respectively. This difference was statistically significant at the $P < .01$ level (Table I). However, lactic acid level in hypoxic adrenalectomized animals was not statistically different from control value of intact animals ($P > .05$). Hypoxic adrenalectomized animals given adrenal hormones as outlined above, had an elevated brain lactic acid content similar to that of the intact hypoxic animals. Adrenalectomized animals given sesame oil only and made hypoxic, had lactic acid values similar to hypoxic adrenalectomized controls (131 *vs.* 136 mg).

Animals which had been maintained at a simulated altitude of 18,000 feet for 12 weeks and were decapitated at that altitude had lactic acid values similar to adrenalectomized hypoxic rats, 137 ± 8 mg *vs.* 136 ± 12 mg. When animals acclimatized to 18,000 feet were placed at 30,000 feet for 105 minutes the

brain lactic acid content was 171 ± 16 mg/100 g of tissue. This value was statistically lower $P < .01$ than that of intact hypoxic brain tissue, and statistically higher $P < .01$ than brain tissue from adrenalectomized hypoxic rats. Altitude acclimatized animals, when returned to ground level for 2 hours, were found to have normal values of brain lactic acid (124 ± 4 mg).

Discussion. These experiments indicate that the adrenal hormones are involved in the accumulation of brain lactic acid following exposure to low oxygen tensions, for when the adrenals were absent, the typical increase in brain lactic acid concentration did not occur. Epinephrine as well as adrenal cortical hormones appeared to be equally effective in the dosages used. Exposure, of intact and adrenalectomized dogs maintained on cortisone or DCA, to a simulated altitude of 30-40 thousand feet, resulted in a moderate increase in blood sugar(6). If the same response occurred in rats in the present study, it may be concluded that the presence of this lactic acid precursor enabled the increase in brain lactic acid to occur following death in the intact and adrenalectomized animals treated with adrenal hormones. Exposure of adrenalectomized rats to hypoxia elevated the brain lactic acid level above that of adrenalectomized non-hypoxic rats which further supports the concept that exposure to hypoxia increased lactic acid precursor; thereby causing an elevated lactic acid level.

Acclimatized animals brain lactic acid level was essentially the same as in non-acclima-

tized animals after being returned to ground level and sacrificed. When killed at altitude to which the animal was acclimatized, however, brain lactic acid was significantly elevated over ground level control and when killed at higher altitude the brain lactic acid increased still further but was considerably below the level found in intact non-acclimatized animals exposed to the same altitude. It appears that acclimatization changed the usual response to altitude as far as brain lactic acid was concerned, probably by altering the lactic acid precursor level of the blood.

Summary. In this study, adrenalectomy prevented a rise in lactic acid content of brain tissue in hypoxic rats. When various adrenal hormones were injected, the brain lactic acid level of adrenalectomized hypoxic rats was elevated to the same extent as intact hypoxic rats. Animals adapted to a simulated altitude

of 18,000 feet did not respond to the same degree as non-adapted animals when both groups were exposed to severe hypoxia.

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Labelling of Antibodies by 5-Dimethylamino-1-Naphthalene Sulfonyl Chloride, Its Effect on Antigen-Antibody Reactions. (23959)

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Histochemical technics using antibodies (Ab) labelled with fluorescent dyes have gained increasing application to immunological problems(1,2). The present paper deals with 5-dimethylamino-1-naphthalene sulfonyl chloride (DNS),† a fluorescent dye recently introduced by Clayton(3). Investigation of the usefulness of this compound for visualization of Ag-Ab reactions was undertaken because of the simple procedure for labelling proteins and the increasing knowledge about the nature of the protein-DNS union(4,5). An enzyme-antienzyme system, yeast alcohol dehydrogenase (ADH)-rabbit antiserum(6,7), was chosen to determine whether labelling of Ab with DNS has any influence on formation of Ag-Ab complex.

Methods. Chemical properties. DNS is a

yellow substance which emits yellow fluorescence. It is almost insoluble in water, but readily soluble in organic solvents like ethanol, ether, ethyl acetate, acetone and chloroform. The corresponding sulfonic acid is formed slowly by hydrolysis but the dye can be used for a few months when kept dry and in the cold. The protein-DNS complex, however, is more stable. Labelled proteins can be precipitated with alcohol, acetone and ammonium sulfate without splitting of the protein-DNS bond. DNS and conjugates absorb light in 300-400 m μ with maxima about 320 to 355 m μ according to medium, pH and combination with different proteins. *Conditions for labelling proteins and DNS-protein ratio.* Proteins buffered to pH 7.4-8.0 are readily labelled by mixing with appropriate amount of DNS dissolved in acetone(4) or ethanol (Vinograd)‡. The suspended unbound dye can be removed by centrifugation while dis-

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† Supplied by California Fn. for Biochemical Research.

‡ Personal communication.

TABLE I. Binding Capacity of Serum Gamma-Globulin and Albumin for DNS.

Protein	DNS	Quotient: $\frac{\mu\text{M DNS bound}}{\mu\text{M protein}}$	
		γ -globulin	Albumin
μM			
.105	3.71	4.1	7.3
.210	"	3.8	7.5
.525	"	3.2	7.0

solved unbound dye may be separated by dialysis or more speedily (together with some dye which may be bound by adsorption) by precipitation of the proteins, as mentioned above, and resolution of washed precipitate. Experiments were performed with purified human gamma globulin (Lederle) and crystallized bovine albumin (Pentex), to measure the quantitative ratio in binding of proteins and DNS. A constant amount of DNS (0.1 ml of a 1% solution in ethanol) was mixed with 16.5, 33.0, and 82.5 mg gamma-globulin and with 7.3, 14.6, and 36.5 mg albumin dissolved in 5 ml phosphate buffer M 0.05, pH 7.4. After 5 minutes at room temperature, samples were immersed in ice bath for 30 minutes. The labelled protein was precipitated with 11.5 ml acetone and samples centrifuged at 5°C for 10 minutes (10,000 x g). The clear yellow supernatant was measured in a spectrophotometer at 366 m μ against dye standards treated in a similar way. The molar ratios of DNS bound to the tested proteins were calculated on the basis of M.W. 156,000 for gamma-globulin and 69,000 for albumin respectively. The observed differences in degree of labelling of albumin and gamma globulin are due to differences in binding sites which appear to be basic groups (most likely primary amino groups) imbedded in lipophilic environment(4,8). From reported data the conclusion can be drawn that all protein molecules present in the experiments are labelled under the stated conditions. The degree of labelling depends on the optimal concentration of DNS. *Reaction rate of Ag-Ab complex formation with labelled Ab.* The use of the ADH-rabbit antiserum system offers a satisfactory method for measurement of Ag-Ab reaction rates. Inhibition of enzyme, as measured by optical test of Warburg(9), corresponds to an early phase of the Ag-Ab

combination. Any factors capable of exerting a specific action on this stage of the Ag-Ab reaction should be detectable (a thorough study of the use of this method for measurement of Ag-Ab reactions will be published). Serum of rabbits immunized with crystalline ADH was used as source of Ab. A partly purified globulin fraction of antiserum was obtained by precipitation with 40% saturated ammonium sulfate and dialysis against saline. The globulin fraction and a corresponding globulin fraction from an unimmunized animal were labelled with an excess DNS as described above. Beckman cells were filled with 2.3 ml phosphate buffer 0.06 M, pH 7.4 (containing 0.02 M semicarbazide), 0.1 ml diphosphopyridine nucleotide (c = 15 mg/ml), and 0.1 ml ethanol (50%). After addition of 0.05 ml Ab fraction or normal globulin fraction, both labelled and unlabelled (each containing 0.55 mg protein), the reaction was started with 4 μ g ADH, and the increase in optical density at 340 m μ was recorded every 15 seconds for 120 seconds. The relative enzyme activity is expressed by the increase in optical density (D) in stated time interval, while inhibition is expressed in per cent of corresponding activities of tests with Ab and blank without Ab

$$\left(100 - D_{\text{Ab}} \frac{D_{\text{control}}}{100} \right).$$

Results. The increased inhibition in time interval 60-90 sec. compared with that of 45-60 sec. shows progressive formation of Ag-Ab complex. This reaction is completed to about 95% within 5 min. after addition of Ag to Ab. As indicated in Table II, there is practically no difference in reaction rate with

TABLE II. Reaction Rates of Ag-Ab Complex Formation (ADH-anti-ADH System) with Labelled and Unlabelled Ab.

Test	Optical density	
	Δ 45-60 sec.	Δ 60-90 sec.
Normal globulin unlabelled	.063	.115
Ab " " "	.038	.066
Inhibition	(39.7%)	(42.7%)
Normal globulin labelled	.063	.115
Ab " " "	.040	.067
Inhibition	(36.5%)	(41.8%)

labelled and unlabelled Ab. In another set of experiments, varying concentrations of DNS were added to tubes containing 4.5 mg purified Ab globulin. Enzyme inhibition by Ab was measured against samples containing similarly treated normal globulin fraction. The results are presented in Table III.

Enzymatic tests with the ADH-anti ADH system demonstrate specific response in an Ag-Ab reaction. These are performed in an excess of Ab within the range of about 1 Ag molecule to 50 Ab molecules. An effect of DNS on the enzyme itself could be an interfering factor in this type of experiments, however, unspecific DNS effects do not occur when blank tests are performed in which a normal globulin fraction is treated exactly as the Ab fraction. Labelling of the enzyme (Ag) can be prevented when the free suspended DNS is separated by centrifugation and precipitation of the Ab preparation. The dissolved free DNS has no measurable effect on activity of the enzyme, neither has the DNS bound to other proteins, but ADH is rather markedly inhibited (up to 75%) when labelled directly with excess of DNS. This inhibitory action may be similar to the effect which DNS exerts on chymotrypsin(10). All differences in inhibition of enzyme activity by labelled and unlabelled Ab in Table III are in the range of experimental error. It appears that DNS has no effect on rate of Ag-Ab reaction.

Ag-Ab precipitate formation with labelled Ab. To investigate the influence of DNS labelled Ab on formation of precipitate, Ag-Ab mixtures were incubated at 7°C for 24 hours; sediment and supernatant were analyzed by biuret reaction. The molecular ratio of 1 Ag: 3 Ab molecules in the precipitate was found to characterize the equivalence zone. Experiments were performed with labelled and unlabelled Ab, using different protein concentrations, but keeping Ag-Ab ratio within the

TABLE III. Reaction Rates of Ag-Ab Complex Formation. Ab labelled with varying DNS concentrations.

DNS added (mg)	.3	.5	.8
% ADH inhibition by Ab (time 45-60 sec.)	36.7	36.7	36.2 40.0

TABLE IV. Total Protein in ADH-Anti-ADH Precipitate Using Labelled and Unlabelled Ab.

		Precipitate (mg protein)		
Labelled	Ab	.640	.388	.198
Unlabelled	"	.673	.404	.180

equivalence zone. Ab protein concentrations were 4.75, 10.0 and 16.4 mg, and corresponding ADH concentrations 0.046, 0.096 and 0.158 mg respectively. The results are given in Table IV. The analysis provides evidence that labelling of Ab does not influence amount of precipitate formed in the ADH-anti-ADH system.

Summary and conclusion. Properties of the fluorescent dye DNS for labelling proteins have been described. Investigations of conditions of DNS binding to serum proteins revealed ratios of 3-4 M DNS/M gamma-globulin and about 7 M DNS/M albumin. Dealing with the use of DNS in immunochemistry, experiments have been carried out with the ADH-rabbit antiserum system, measuring enzyme inhibition by the Ab. Labelling of Ab with DNS did not affect rate of formation of Ag-Ab complex and had no influence on quantity of formed precipitate. The reported data suggest that because DNS does not affect the Ag-Ab reaction, it can be successfully applied in histochemical studies.

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Liver Arginase, Histidase and Phenylalanine Transaminase Following Administration of their Respective Substrates.* (23960)

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Several enzyme activities that catalyze the first step in catabolism of certain amino acids increase after administration of their substrates, for example, tryptophan peroxidase-oxidase(1), tyrosine (α -ketoglutarate) transaminase(2), phenylalanine (α -ketoglutarate) transaminase (E.C.C. Lin, personal communication) and threonine dehydrase(3). Arginase activity increases when the protein content of the diet is increased(4,5,6) although feeding of arginine itself in high amounts does not alter this enzyme level(7). L-Amino acid oxidase levels can also be increased by administration of amino acids(8). Koizumi(9) administered large doses of L-histidine to rabbits prior to isolation of histidase. No reason was given for this procedure, which led Knox (1) to the discovery of adaptive nature of tryptophan peroxidase-oxidase. We recently observed(10) that phenylalanine (pyruvate) transaminase activity of livers of leukemic rats was directly related to the blood level of phenylalanine. Experiments were designed to ascertain whether arginase, histidase and phenylalanine (pyruvate) transaminase adapt to their respective substrates, since this would be of considerable theoretical interest.

Materials and methods. Albino rats (Holtzman) weighing 100 g (250-300 g in the arginase experiment) and white or Himalaya rabbits weighing approximately 2 kg were used. Test substance was administered *via* intraperitoneal injection in the following dosages: L-arginine in solution adjusted to pH 7, 0.75 mmole/100 g body weight; L-histidine in solution adjusted to pH 7, 1 g/kg body weight of rat and 0.5 g/kg body weight of rabbit; L-phenylalanine 0.5 mmole/100 g body weight. This dose of histidine was the same as that used by Koizumi(9), and the

dose of phenylalanine was sufficient to increase plasma phenylalanine by 100%, an amount comparable to elevation observed in leukemic rats(10). The arginine dosage was chosen to fall within this same general range, *i.e.* 0.3 to 1.0 mmole/100 g body weight. Five hours after injection, all animals were decapitated. Livers of normal and treated animals were excised, weighed, and 5 g portions were homogenized for 2 minutes in Waring blender, fitted with stainless steel micro-head, with 7 volumes of 0.14 N KCl containing 0.0025 N NaOH. Homogenates were centrifuged in the high speed attachment of International Refrigerated Centrifuge, Model PR-2, for 45 minutes at 25,000 $\times$ g. The supernatant fractions were then analyzed for enzyme activity. Arginase activity was determined at pH 9.1 in the presence of 2.5×10^{-4} M Mn^{++} ion. The urea produced was determined according to Archibald(11). Histidase activity was measured by direct spectrophotometric method of Tabor and Mehler(12). Phenylalanine (pyruvate) transaminase assays were carried out using borate-arsenate method of Lin(13). All assays were performed at 38°C. Dry weights of homogenates, corrected for KCl in the homogenizing fluid, were obtained on 5 ml samples dried for 2 hours at 110°C in tared aluminum moisture dishes. Data are expressed in terms of μ moles product formed/hr/g dry wt.

Results. It is readily apparent from the data of Tables I and II that none of the enzymes tested responded with any significant change to administration of their respective substrates. Changes in arginase activity as a function of protein intake of animals(4,5,6) may be a reflection of stimulation of the urea cycle with increased nitrogen load. However, it is known that of all enzymes of the urea cycle, arginase is present in amounts which are not rate-limiting(14). It is possible that injection of histidine in Koizumi's work(9)

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TABLE I. Liver Histidase following Histidine Injection.

		Histidase, μ moles urocanate/g/hr
Rat	Control, ♂	54.0 (3)*
	Treated, ♂	52.1 (4)
	Control, ♀	50.4 (3)
	Treated, ♀	45.0 (3)
Rabbit	Control, ♂	12.4 (1)
	Treated, ♂	9.6 (2)
	Control, ♀	14.8 (2)
	Treated, ♀	10.0 (1)

* Values in parentheses indicate No. of animals used.

led to stabilization of the enzyme. The lack of change in the phenylalanine (pyruvate) transaminase activity following phenylalanine administration indicates that the observed changes of this enzyme in leukemic rats(10) were not a result of changes in the circulating phenylalanine levels but were the direct consequences of the leukemic state.

It would thus appear that although the mechanism of substrate-induced adaptation of certain amino acid catabolizing enzymes may be the means by which certain of the circulating amino acid levels are regulated, this mechanism cannot be the one which is gen-

TABLE II. Liver Arginase and Phenylalanine (Pyruvate) Transaminase following Arginine and Phenylalanine Injection.

		Phenylalanine (pyruvate) transaminase, μ moles phenyl- pyruvate/g/hr
Rat	Arginase, μ moles urea/g/hr	
Control, ♂	61,300 (3)*	218 (3)
Treated, ♂	73,600 (3)	207 (4)

* Values in parentheses indicate No. of animals used.

erally used by the animals for homeostatic regulation of all circulating amino acid levels, or we would have observed adaptive changes in the arginase, histidase and phenylalanine transaminase levels after the administration of their respective substrates.

Summary. Rat liver arginase, histidase and phenylalanine (pyruvate) transaminase as well as rabbit liver histidase did not increase following injection of their respective amino acid substrates.

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Survival of Mouse-Grown Rat Erythrocytes. (23961)

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Results of several lines of investigation have shown that irradiated mice injected with foreign hematopoietic cells accept the new tissue to the extent that this tissue implants, proliferates, and functions in the irradiated recipient (see review by Congdon,1). For example, in one experimental situation, Makinodan(2) using immunohematological technics, observed that the erythrocytes of X-irradiated mice injected with rat bone marrow were of rat origin. Furthermore, certain physicochemical properties of these mouse-grown rat erythrocytes were more like those of a rat than those of a mouse(3). To investigate a physiological characteristic of the mouse-grown rat erythrocyte, we used $\text{Na}_2\text{Cr}^{51}\text{O}_4$ as the label to compare survival time of these transplanted cells with survival time of normal rat and normal mouse erythrocytes.

Materials and methods. In all the experiments, (101 X C3H) F_1 mice and Sprague-Dawley rats were used. Mouse-grown rat erythrocytes were obtained from mice that had received 950 r of total-body X radiation followed by an injection of rat bone marrow cells. Erythrocytes of mice that survived at least 60 days were tested against species-specific antirat serum, and only those mice with 100% circulating rat erythrocytes, according to the test, were used. The $\text{Na}_2\text{Cr}^{51}\text{O}_4$, obtained from Abbott Laboratories, had a specific activity of 20-40 mc/mg. Blood for transfusions was aspirated from the heart of donor animals into heparinized syringes. The $\text{Na}_2\text{Cr}^{51}\text{O}_4$ was added to the blood and the mixture incubated at room temperature for 1 hour. Whole blood containing the labeled erythrocytes was injected into the jugular or tail vein of the recipient animal. The initial 24-hour blood sample was obtained from the tail vein or the heart, the erythrocytes lysed

in 1.0 ml of distilled water, and radioactivity of the sample determined in a well-type scintillation counter. The 24-hour sample was taken as 100% and radioactivity of subsequent samples plotted as the percentage of this activity. Survival times in rats of normal rat, normal mouse, and mouse-grown rat erythrocytes were determined. These erythrocytes were labeled with $\text{Na}_2\text{Cr}^{51}\text{O}_4$ (8-15 $\mu\text{C}/\text{ml}$ of whole blood), and 1 ml of the labeled blood was injected into recipient rats. Tail blood (20- μl samples) was obtained from each of these rats at 24 hours and subsequently at various intervals up to 60 days. Hematocrits were determined concomitantly. Two methods were used in studying the survival time in mice of normal rat, normal mouse, and mouse-grown rat erythrocytes. In one method, recipient mice were injected with 0.3 ml each of whole blood containing erythrocytes labeled with $\text{Na}_2\text{Cr}^{51}\text{O}_4$ at concentrations of 10 or 30 $\mu\text{C}/\text{ml}$ of whole blood. The usual serial sampling method was then used whereby small amounts of blood (10 μl) were obtained from the tail of each mouse at various intervals after injection of the labeled erythrocytes. The other method involved injection of 25-35 mice with 0.1 ml each of labeled whole blood (incubated with 4.8-8.0 μC of $\text{Na}_2\text{Cr}^{51}\text{O}_4/\text{ml}$ of whole blood). At various intervals thereafter a group of 4 or 5 of these mice was sacrificed to obtain 0.5 ml of heart blood from each animal. When this group method was used on mice of the same age, sex, and weight (within 2 g of each other), variation in blood-sample radioactivity among mice within any one group was less than 10%. Two points on each curve were used as a measure of erythrocyte survival. The time when 50% of the initial radioactivity remained is referred to as the 50% survival, and the intercept of the curve with the 5% radioactivity level was taken as an estimate of the maximum erythrocyte life span. We felt that, for our purposes, the 5% radioactivity level was better than the inter-

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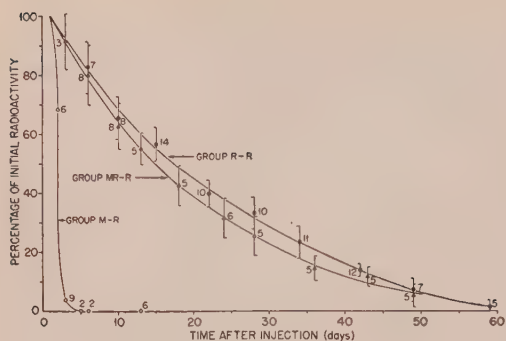


FIG. 1. Survival of normal rat, mouse-grown rat, and normal mouse erythrocytes in the rat, as determined by Cr^{51} labeling. R-R = rat RBC in rat; MR-R = mouse-grown rat RBC in rat; M-R = mouse RBC in rat. Vertical bars indicate $\pm$ one stand. dev. Figures beside points indicate No. of animals.

cept of the curve with the abscissa because of the asymptotic approach of the curve to the zero radioactivity level.

Results. Erythrocyte survival in rats (Fig. 1). The 50% survival for normal rat erythrocytes in the rat (group R-R) was 18 ± 2.5 days,[†] and the 5% survival was 52 ± 4.5 days. The 50% survival for mouse-grown rat erythrocytes in the rat (group MR-R) was 15 ± 2.5 days, and the 5% survival was 51 ± 4.5 days. The 50% survival for normal mouse erythrocytes in the rat (group M-R), however, was approximately 2 days, and the 5% survival was approximately 3 days; no detectable radioactivity was found on the 13th day. Although the 50% survival of group R-R was slightly greater than that of group MR-R, a comparison of the 2 slopes (log plot of the radioactivities from day 1 to day 49) of the groups indicated that there was no significant difference between the 2 survival curves. The slopes and standard errors of groups R-R, MR-R, and M-R were -0.020 ± 0.003 , -0.023 ± 0.004 , and -0.248 ± 0.125 respectively. The slope for group M-R was significantly greater ($P < 0.001$) than the slopes for groups R-R and MR-R. Hematocrits for all recipient rats remained at approximately control levels throughout the course of the experiments.

Erythrocyte survival in mice (Fig. 2). The data from both the serial and the group sam-

pling methods were combined to construct the curves. The 50% survival for normal mouse erythrocytes in the mouse (group M-M) was 20 ± 2.5 days, and the 5% survival was 49 ± 5.0 days. The 50% survival for normal rat erythrocytes in the mouse (group R-M) or mouse-grown rat erythrocytes in the mouse (group MR-M) was approximately 2 days, and the 5% survival was 3-4 days. In groups R-M and MR-M no radioactivity was detected in samples obtained on days 9 and 16. Hematocrits of the recipient mice were essentially unchanged during the course of the experiments.

Discussion. The survival time of the mouse-grown rat erythrocytes in the rat was not significantly different from that of normal rat erythrocytes. This indicates that, by this physiological criterion, the transplanted erythrocytes are compatible with a rat environment and are therefore of rat origin. If they were of mouse type, their maximum life span would approach or be equal to that of the normal mouse erythrocyte in the rat; namely, 3 days. These results confirm those reported by Makinodan(2) and Vos *et al.*(4), who found that the mouse-grown rat erythrocytes agglutinate with antirat erythrocyte serum but not with antimouse erythrocyte serum.

When the mouse was used as a recipient for the labeled cells, we observed that the maximum life span of mouse-grown rat erythrocytes was the same as the maximum life span of normal rat erythrocytes; namely, 3-4 days.

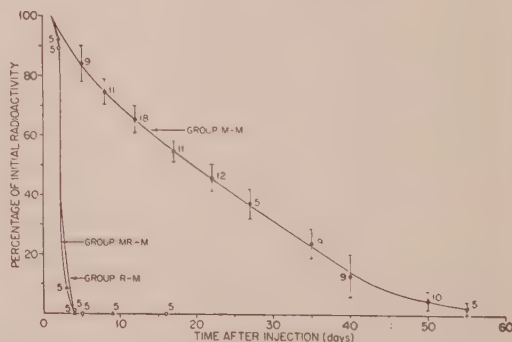


FIG. 2. Survival of normal mouse, mouse-grown rat, and normal rat erythrocytes in the mouse, as determined by Cr^{51} labeling. M-M = mouse RBC in mouse; MR-M = mouse-grown rat RBC in mouse; R-M = rat RBC in mouse. Vertical bars indicate $\pm$ one stand. dev. Figures beside points indicate No. of animals.

[†] The figures after survival times are 1 stand. dev.

These results further show that the mouse-grown rat erythrocytes are of rat type; if they were of mouse origin, their survival time would be similar to that of normal mouse erythrocytes.

In these experiments, the shapes of the survival curves for the compatible erythrocytes were curvilinear. Using the Cr^{51} method, other investigators have obtained similarly shaped curves for erythrocytes of man and several laboratory animals (5-9). Factors that might contribute to the shape of a survival curve include the elution of Cr^{51} from erythrocytes, normal or abnormal random destruction of the erythrocytes, and a relatively short mean survival time coupled with a large variation in survival of individual erythrocytes (see reviews by Eadie and Brown, 10 and Dornhorst, 11). Reutilization of the isotope could also affect the shape of a survival curve, but the work of Ebaugh *et al.* (5) indicates that Cr^{51} does not reincorporate into circulating human erythrocytes to an appreciable extent. It is difficult to ascertain the influence of these factors on erythrocyte survival curves. The *in vivo* elution of Cr^{51} from erythrocytes can be implicated if the Cr^{51} method is compared with a second method known to produce a linear line resulting from senescence of the erythrocytes. This comparison can be made for human erythrocytes by simultaneous use of the Ashby and Cr^{51} technics (5,12).

Comparison of the shapes of the survival curves for normal mouse and normal rat erythrocytes shows less curvilinearity for the mouse than for the rat erythrocytes. Several factors could contribute to this difference: (a) elution rate of Cr^{51} from mouse erythrocytes may be less than that for rat erythrocytes, (b) variation in the life span of individual mouse erythrocytes may be smaller than variation in life span of individual rat erythrocytes, (c) the mice used were more inbred than the rats and, therefore, transfused erythrocytes from one mouse to another might be more compatible than transfused erythrocytes from one rat to another, and (d) random destruction of erythrocytes may be greater in the rat than in the mouse.

Summary. A study was made of survival

of Cr^{51} -labeled erythrocytes from irradiated mice that had been given rat bone marrow. Survival of these erythrocytes (mouse-grown rat erythrocytes) in both mice and rats was compared with survival of normal rat and normal mouse erythrocytes in mice and rats. The 50% survival time, *i.e.* the time when 50% of the initial radioactivity remained, of mouse-grown rat erythrocytes in the rat was 15 ± 2.5 days, and the 5% survival time, *i.e.* the time when 5% of the initial radioactivity remained, was 51 ± 4.5 days. Respective values for survival of normal rat erythrocytes in the rat were 18 ± 2.5 and 52 ± 4.5 days and for normal mouse erythrocytes in the rat, 2 and 3 days. The 50% survival time of mouse-grown rat and of normal rat erythrocytes in the mouse was 2 days and the 5% survival time was 3-4 days. Respective values for the survival of normal mouse erythrocytes in the mouse were 20 ± 2.5 and 49 ± 5.0 days. The data show that, by one physiological criterion, erythrocytes of X-irradiated mice (950 r) given rat bone marrow are of rat origin since their survival time in the rat is essentially the same as the survival time of normal rat erythrocytes in the rat.

The authors are grateful to Drs. C. C. Congdon and T. Makinodan for their help and interest during the course of this investigation, and Dr. N. Gengozian for determining the percentage rat erythrocytes in the irradiated donor mice that had received rat bone marrow.

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Changes in Cerebral Potassium During Insulin Hypoglycemia.* (23962)

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Little is known about metabolism of cerebral potassium during insulin hypoglycemia. Most recently La Fran *et al.*(1) have postulated that electroencephalographic changes and alterations of consciousness observed in both acute and prolonged insulin coma are in part due to shifts in electrolytes and water within the brain. They have postulated a decrease in intracellular potassium as a result of the deposition of brain glycogen. This results in a distortion of the intra- and extracellular relationship of potassium and sodium giving rise to extracellular edema. Several investigations are not in accord with this hypothesis, since it has been shown that there is an increase in intracellular potassium in liver, muscle, and erythrocytes during hypoglycemia(2-6). Further, Himwich has demonstrated a drop in cerebral glycogen content during hypoglycemia(7). Finally, *in vitro* and *in vivo* studies of the effect of insulin on cerebral metabolism have not demonstrated increased glucose utilization(8-10). These studies would tend to refute the arguments offered by La Fran *et al.*(1) to support their hypothesis, and would indicate that the changes observed electroencephalographically and clinically are primary or secondary results of a lowered level of metabolic substrate. This study was undertaken in an effort to clarify if possible, the changes that might be observed in the total brain potassium following a marked lowering of the blood sugar.

Method. 12 adult white male rats of the Osborne-Mendel strain were given 50 units of 80

units/cc crystalline regular insulin intravenously in a tail vein. The rats were then sacrificed by decapitation at 1½ to 5½ hours after injection, and in varying depths of hypoglycemic reaction ranging from stupor to coma. The whole brain including cerebellum was then analyzed for potassium by modification of the method of Elliott and Jasper(11). Following determination of wet and dry weights, samples were ashed in an electric furnace at 650°C for 4 to 5 hours and remained overnight at 350° to 400°C(11). The ashed weight was determined. The samples were then dissolved in 10 cc of concentrated hydrochloric acid, diluted to 100 cc with distilled water. Potassium was determined using an internal standard flame photometer (12). 12 control rats were lightly anesthetized with ether, decapitated, and the above procedure was carried out.

Results. In comparing brains obtained from experimental and control groups, there appeared to be no observable edema in either group; on close inspection, however, it was noted that in the insulin-treated group the brains appeared to be more friable, hyperemic and glistening. Also, the meninges were more adherent to the surface of the cortex.

There was no statistically significant increase in water content of brains of hypoglycemic rats. The values obtained were: controls 78.16%, hypoglycemic 78.38% ($p = >.50$).

Comparison of the amount of potassium on the basis of wet brain showed no statistically significant difference. The values were: control 92.89 meq. potassium/kg, hypoglycemic

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TABLE I. Cerebral Potassium Levels during Hypoglycemia.

	Mean	S.D.	t	p
<i>meq K/kg</i>				
Wet wt control	92.89	6.11	1.07	.30
Insulin	95.45	3.08		
Dry wt control	425.8	25.0	2.19	<.05
Insulin	446.2	9.0		
Ash wt control	6158.0	719.0	2.04	>.05
Insulin	6726.0	344.0		
<i>% water</i>				
Control	78.16	.55	.37	>.50
Insulin	78.38	1.67		

95.45 meq. potassium/kg ($p = .30$).

In contrast to the above negative findings there was a statistically significant increase in dry grain potassium. These findings were: controls 425.8 meq. potassium/kg, hypoglycemic 446.2 meq. potassium/kg ($p = < 0.05$). The ash weight values showed a near statistically significant increase ($p = > 0.05$). These values were: controls 6158.0 meq. potassium/kg, hypoglycemic 6726.0 meq. potassium/kg.

Discussion. The data show a statistically significant increase in dry brain potassium. Although values for potassium in the ashed samples were not significant, it is possible that the combination of a near significant value for ashed brain plus a small increase in water content combined to give a significant dry weight value. The distribution of potassium in the brain is not known, but it may be assumed that the increase in cerebral potassium is on the basis of increase in intracellular potassium, since the intracellular values have been estimated to be approximately 99 times those of the extracellular fluid(13). It is obvious that there would have to be a large increase in the extracellular potassium to account for these changes.

Previous investigators have demonstrated, *in vitro*, promotion of glycogenesis by potassium. This glycogenesis and metabolism of glucose is facilitated by insulin. The knowledge that 0.36 meq. of potassium is bound with each gram of glycogen and is released by glycogenolysis would explain, at least in part, the transport of potassium into an intracellular position in the brain(14). However, to

date, no evidence, either *in vitro* or *in vivo* has been accumulated to demonstrate a facilitation of cerebral glucose metabolism by insulin(12-13).

Finally, it must be pointed out that the results of this study raise the question of the influence of insulin on cerebral glucose utilization. The significant increase in potassium seen here is similar to that produced in other tissues when glucose metabolism is stimulated by insulin. In view of the fact that most studies of insulin coma have been done in the hypoglycemic state it would seem necessary to study the effects of insulin on glucose metabolism of the brain where an adequate supply of substrate was available.

Conclusions. 1. A significant increase in cerebral potassium levels of dry brain was demonstrated in insulin-treated rats. 2. Hypoglycemia had no significant effect on water content of brains. 3. The importance of this finding as related to the present concept of cerebral glucose metabolism is discussed.

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Anticoagulant Action of Protamine Sulphate.* (23963)

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It has been known for a long time that protamine sulphate has an anticoagulant action (1,2). Ferguson(3) showed that protamine sulphate retarded or prevented conversion of prothrombin to thrombin in presence of brain but accelerated formation of fibrin from fibrinogen by thrombin; he called the latter action fibrinoplastic. In higher concentrations protamine precipitates fibrinogen(3,4) and it has been suggested that this is its principal anticoagulant effect(4); this reaction is reversible(5). Tocantins(6) showed that protamine sulphate prolonged the one-stage prothrombin time and believes that it is a true antithromboplastin as well as antiprothrombic. Portmann and Holden(7) however were unable to demonstrate an antithromboplastic action of protamine. The following study was undertaken to analyse the fundamental mechanisms involved in the inhibitory action of protamine sulphate with particular respect to blood thromboplastin generation.

Materials and methods. *Thromboplastin generation test (TPG)* was performed by the technic of Biggs and Douglas(8). $Al(OH)_3$ adsorption was performed by the method of Biggs and Macfarlane(9). *Serum factors* adsorbed by this technic were eluted with phosphate buffer (pH 8); this eluate is relatively free of inhibitors(10). *AHF* was prepared by the 33% saturation of $Al(OH)_3$ treated plasma(9), this material also contains fibrinogen and some factor V but is relatively free of antiaccelerator activity(11). *Partial thromboplastin times (PTT)* were performed by the method of Langdell, *et al.*(12), modified by the addition of protamine sulphate immediately before the addition of the final calcium. *One-stage prothrombin times* were determined by mixing 0.1 ml normal plasma, 0.1 ml fresh

thromboplastin solution (Difco), 0.1 ml protamine sulphate solution and 0.1 ml 0.025M $CaCl_2$ in the order named. *Thrombin-fibrinogen times* were performed by adding 0.1 ml of bovine thrombin (10 units/ml) to a mixture of 0.2 ml of the 33% ammonium sulphate fraction of $Al(OH)_3$ -treated plasma and 0.1 ml protamine sulphate or saline; the protamine sulphate was added 5 seconds before the final addition of thrombin. *Protamine sulphate* (Lilly) was used and diluted with saline buffered with imidazole (pH 7.2). Concentrations of protamine sulphate in all instances refer to concentration of protamine sulphate in the final clotting mixture except when otherwise stated.

Results. *Effect of varying concentrations of protamine sulphate on one-stage prothrombin time, PTT and TPG.* The PTT test is a slightly more sensitive index of the anticoagulant action of protamine sulphate than the one-stage prothrombin time and TPG test in respect of maximum yield (reflected by minimal substrate clotting time) (Table I). The protamine sulphate however was found to diminish the rate as well as the yield of blood thromboplastin formation although the action

TABLE I. Effect of Protamine Sulphate on Partial Thromboplastin Time (PTT), Thromboplastin Generation Test (TPG), One-Stage Prothrombin Time (1-Stage PT) and Thrombin-Fibrinogen Time.

Cone. protamine sulphate in incubation mixture, μg/ml	PTT	TPG, min substrate clotting times	Sec.	
			1-stage PT	Thrombin- fibrinogen time
125	200	32	41	Floc.*
62.5	140	21	32	5
25	125	16	25.5	6
15	108	15	21.5	7.2
7.5	102	13	19.0	8
3	98	9.8	17.8	8.1
2	93	8.3	16.2	8
1.5	90	8.0	15.2	8.2
1	82	8.1	15.4	8.2
.5	79	8.0	15.1	8
Saline control	80	8.2	15.2	8

* Flocculation.

* This investigation was supported by grant H-3536 from Nat. Heart Inst., N.I.H., Public Health Service.

Since submitting this paper very similar experimental findings were reported at Annual Meeting of Fed. Soc. for Exp. Biol. and Med., April, 1958 at Philadelphia, by Dr. J. N. Shanberge.

TABLE II. Effect of Protamine Sulphate on Rate of Thromboplastin Generation.

Cone. protamine sulphate in incubation mixture, $\mu\text{g/ml}$	Min substrate clotting time, sec.	Period of incubation required for min time, min.
Saline control	8.1	3
2	8.5	4
3	8.3	7
7.5	7.9	9
15	9.4	13

on rate was not always apparent due to normal deterioration of fully formed blood thromboplastin after several minutes. The effect on the rate was best brought out by substituting serum eluate and 33% ammonium sulphate fraction for normal serum and $\text{Al}(\text{OH})_3$ treated plasma in the TPG test. This test system is relatively free of inhibitors and deterioration of fully formed thromboplastin is delayed. Using this system (Table II) it was found that concentrations of protamine sulphate in the incubation mixture ranging from 7.5 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$ which was the lowest effective concentration, diminished rate but not yield of blood thromboplastin formation. Higher concentrations affected both yield and rate. In further experiments it was shown that the addition of extra amounts of $\text{Al}(\text{OH})_3$ -treated plasma, serum or platelets to the TPG test diminished the inhibitory effect of the protamine sulphate but these findings are difficult to evaluate since the control TPG mixture containing saline instead of protamine sulphate also showed increased coagulant activity. Ferguson† has found that BaCO_3 adsorbed beef serum diluted 1 in 10 has an antagonistic effect on the action of protamine in respect to the one-stage prothrombin time. This work was confirmed and it was also found that adsorbed beef serum would correct the defect in the TPG test produced by protamine sulphate.

Effect of preincubation of protamine sulphate with formed blood thromboplastin. A thromboplastin generation test was set up using 1 ml of each of the following reagents in the usual concentrations: $\text{Al}(\text{OH})_3$ -treated plasma, serum, platelets and calcium chloride. When the coagulant activity of the mixture

reached a maximum, it was divided into 2 equal 1 ml parts. To one of these was added 0.2 ml of saline and to the other the 0.2 ml of protamine sulphate to give a final concentration of 7.5 $\mu\text{g/ml}$, this concentration of protamine had been previously shown to produce a significant diminution of yield of blood thromboplastin (minimum substrate clotting time of 15 seconds with a normal control of 10 seconds) when preincubated with the TPG test reagents used in this particular experiment. Immediately before and after the addition of protamine or saline and at subsequent 5 minute intervals samples were removed from each of the 2 mixtures and tested for coagulant activity. No significant differences between the two systems were obtained; the minimum substrate clotting times 1 minute after the addition of protamine sulphate or saline were both 10 seconds, showing that concentrations of protamine sulphate sufficient to produce marked inhibition on thromboplastin generation may have no effect on fully formed blood thromboplastin. The rate of deterioration was the same in both cases. If higher concentrations of protamine were added to the TPG mixture after the development of its maximal coagulant activity, the subsequent coagulant activity was less than that of the saline control but the difference was never appreciable. Thus, in one experiment a substrate clotting time of 13 seconds compared to 11 seconds in the saline control was obtained 1 minute after the addition of the protamine sulphate in a final concentration of 50 $\mu\text{g/ml}$ or saline. Ten minutes later, both mixtures gave a substrate clotting time of 15 seconds, showing that the deterioration in the coagulant activity of the TPG mixture containing the protamine sulphate was not greater than that of the saline control.

Effect of adding varying concentrations of protamine sulphate to the substrate plasma in TPG. A thromboplastin generation test was set up as in the previous experiment using the same reagents but at no stage was protamine sulphate added to the incubation mixture. The coagulant activity of this mixture was tested at minute intervals and when the minimal substrate clotting was obtained the tubes containing substrate plasma were re-

† Personal communication.

TABLE III. Effect of Addition of Protamine Sulphate to Substrate Plasma in Thromboplastin Generation Test after Development of Maximal Activity.

Conc. of protamine sulphate in tube after addition of CaCl ₂ , $\mu\text{g/ml}$	One-stage "prothrombin" times (sec.)		
	Blood thromboplastin	Diluted blood thromboplastin	Brain thromboplastin
125	15	20	36
62	13	17.2	25
25	11	17.2	19.5
15	11	15	17
7.5	11	15	16
3	11	15.5	15
Saline control	11	15	15

placed by tubes containing the same plasma substrate to which had been added saline or varying concentrations of protamine sulphate. The clotting times of these tubes were then determined in rapid sequence by the addition of blood thromboplastin and calcium as in the performance of one-stage prothrombin times. The experiment was concluded before the activity of the fully formed thromboplastin deteriorated. In a similar experiment the fully formed thromboplastin was diluted so as to have a coagulant activity equivalent to an extract of brain thromboplastin and the "one-stage prothrombin" times of mixtures of normal plasma and varying concentrations of protamine sulphate determined as before using the fully formed blood thromboplastin. The same experiment was then repeated using the brain thromboplastin instead of blood thromboplastin. The results (Table III) show that a concentration of protamine sulphate as high as 25 $\mu\text{g/ml}$ has no effect on the "one-stage prothrombin" times performed using fully formed blood thromboplastin although this concentration will prolong the one-stage prothrombin time performed using a conventional tissue thromboplastin. The one-stage "prothrombin times" of both type of thromboplastin were prolonged by concentrations of protamine higher than 25 $\mu\text{g/ml}$.

Effect of preincubation of protamine sulphate with brain extract or plasma on one-stage prothrombin time. A concentration of protamine sulphate sufficient to lengthen the one-stage prothrombin time of normal plasma to 30 seconds was preincubated for varying

periods up to 1 hour with brain. 0.2 ml aliquots of the mixture were removed at intervals and added together with 0.1 ml of .025M calcium chloride to 0.1 ml normal plasma and the clotting time recorded. A parallel experiment was performed using the same reagents but in which the protamine was incubated with plasma, aliquots removed at intervals and recalcified after the addition of brain so that the final clotting system was identical in the two experiments. It was found that the preincubation in both experiments produced no significant change in the clotting times which in all instances varied between 28 and 32 seconds.

Discussion. These findings show that protamine sulphate in relatively low concentrations diminishes both rate and yield of blood thromboplastin; these concentrations have no effect on fully formed blood thromboplastin and do not inhibit the reaction between blood thromboplastin and prothrombin in the substrate clotting tubes in the TPG test. In such low concentrations protamine sulphate is therefore antithromboplastinogenic and not antithromboplastic or antiprothrombic with reference to the action of blood thromboplastin. However low concentrations of protamine sulphate prolonged the one-stage prothrombin time performed by conventional methods as was first shown by Tocantins(6). At present, only factor V, Stuart factor and possibly factor X appear to be essential components of both the one-stage prothrombin and TPG test systems and as protamine sulphate is inhibitory in both these systems, it is possible that it acts specifically against one or more of these coagulation factors. The present status of factor X is now in doubt (13). Stuart factor affects yield rather than rate of blood thromboplastin formation while factor V affects both rate and yield so that on this hypothesis protamine sulphate would be more likely to act against factor V than Stuart factor.

The finding that the anticoagulant actions of protamine sulphate can be neutralized by BaCO₃ adsorbed beef serum which is rich in factor V but believed to be deficient in all other known coagulation factors appears to be further evidence supporting the hypothesis

that protamine sulphate is in fact an anti-factor V but the author agrees with Ferguson (personal communication) that this interpretation is unjustified since such serum is an extremely complex reagent. The unrelated finding that the addition of extra platelets, $\text{Al}(\text{OH})_3$ treated plasma or serum to a normal thromboplastin generation system preincubated with protamine sulphate tend to neutralize the effect of the latter should also be viewed in the same light and interpreted with extreme caution.

It is clear that protamine sulphate has more than one anticoagulant action and several workers have confirmed that very high concentrations cause precipitation of fibrinogen and that relatively lower concentrations have a fibrinoplastic action(3,7). The lowest concentrations of protamine required for the fibrinoplastic action are roughly equivalent to the lowest concentration necessary to interfere with the reaction between blood thromboplastin, plasma and calcium and this concentration is considerably higher than the minimal concentration affecting the yield of blood thromboplastin generation.

The inhibitory action of protamine in high concentrations on the reaction between fully formed blood thromboplastin, plasma and calcium appears an immediate one and is non-progressive. It is possible that this action of protamine is both antiprothrombic and anti-thromboplastic as suggested by Tocantins(6) but other interpretations are possible.

Summary. Protamine sulphate in low concentrations affects both rate and yield of blood thromboplastin but has no effect on formed thromboplastin. Relatively higher concentrations inhibit a reaction between blood thromboplastin, prothrombin and calcium.

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Effect of Chlorothiazide on Vascular Reactivity.* (23964)

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(Introduced by A. Goth)

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Chlorothiazide (Diuril) has been recently introduced as an orally effective diuretic agent (1). Clinical studies(2,3) have indicated a definite anti-hypertensive effect of this compound, and the suggestion has been made that it may have a direct hypotensive action(4). Thus far, however, there has been no indica-

tion of a mechanism whereby chlorothiazide might act to reduce blood pressure directly. Therefore, the present study was undertaken to determine the effect of this substance upon vascular response to certain pressor agents.

Methods. 42 experiments were performed on adult mongrel dogs. They were anesthetized with sodium pentobarbital (30-35 mg/

* Aided by USPH grants.

TABLE I. Effect of Chlorothiazide on Vascular Reactivity.

Dose ($\mu\text{g/kg}$)	Control				Chlorothiazide				Level of significance*
	No. of animals	Before infusion (mm Hg)	After infusion (mm Hg)	Change	No. of animals	Before infusion (mm Hg)	After infusion (mm Hg)	Change	
<i>A. Pressor responses to norepinephrine</i>									
.05	9	17.3	16.7	- .6	8	20.4	12.6	-7.8	<.01
.10	9	24.1	22.7	-1.4	7	26.7	17.8	-8.9	"
<i>B. Pressor responses to epinephrine</i>									
.05	7	9.8	9.7	- .1	14	11.1	7.8	-3.3	<.05
<i>C. Depressor responses to isopropylnorepinephrine</i>									
.05	9	16.0	17.6	+1.6	7	16.7	10.6	-6.1	"

* Considered significant if $P < .05$.

kg), vagotomized, and their arterial blood pressures recorded on a smoked drum by mercury manometer. In the first series, 2 responses to noradrenaline, 0.05 and 0.1 $\mu\text{g/kg}$ were elicited, then 20 mg/kg chlorothiazide in 10 ml of 5% glucose was given intravenously followed by 20 mg/kg chlorothiazide in 60 ml 5% glucose infused over 30 minutes. The norepinephrine injections were then repeated. In a second series, 2 injections of .05 $\mu\text{g/kg}$ epinephrine were given, chlorothiazide infused as in the first group, and epinephrine doses were repeated. A third group of dogs received .05 $\mu\text{g/kg}$ isopropylnorepinephrine (Isuprel) before and after chlorothiazide infusion. Animals treated identically to the experimental groups with the exception that no chlorothiazide was present in the glucose solutions served as controls. The response to noradrenaline and adrenaline was considered to be the difference between mean arterial pressure before injection and highest mean pressure after injection. Similarly the response to Isuprel was determined by difference in mean pressure before administering Isuprel and lowest mean pressure following injection. Responses in all animals were determined from a base pressure which differed less than 10 mm Hg from control levels. Plasma sodium concentrations were measured by means of a Beckman flame photometer.

Results. Chlorothiazide infusion reduced the pressor response to norepinephrine and epinephrine (Table I, A & B). The decrease occurred in all dogs tested with norepinephrine, and failed to occur in only one animal challenged with epinephrine. Chlorothiazide also reduced the depressor response to isopro-

pynorepinephrine in a consistent manner (Table I, C).

The initial responses of experimental and control animals to norepinephrine, epinephrine, and isopropylnorepinephrine were not appreciably different, and the small increases or decreases in responsiveness after glucose infusion were not significant. On the other hand, the differences noted in reactivity before and after chlorothiazide were highly significant and comparisons of quantity changes in response to the 3 amines of the control and chlorothiazide groups were significant (Table I) when compared with each other.

This lowered responsiveness induced by chlorothiazide is somewhat slow in onset, being well developed after a 30 minute infusion, but is not evident 10-15 minutes following intravenous injection of 10 to 20 mg/kg of the material.

Plasma sodium concentrations after chlorothiazide infusions showed a small decrease from control values in 17 dogs averaging 2.4 meq/l, but this decrease did not approach significance when compared with the small plasma sodium variations in control groups. Elevation of plasma sodium by infusion of hypertonic sodium chloride into the chlorothiazide treated animal did not alter the lessened response to norepinephrine.

Discussion. Although a direct hypotensive action of chlorothiazide has been suggested from clinical evidence(4), a mechanism for this effect is lacking. Acute experiments involving infusion of chlorothiazide have shown no depression of blood pressures(2). The present study, illustrating that this diuretic agent decreases the pressor response to nore-

pinephrine and epinephrine, may shed light upon the clinically observed antihypertensive effect.

The possibility that chlorothiazide is a weak adrenergic blocking agent appears unlikely since its effect is not greater in diminishing the response to epinephrine than to norepinephrine and further since it also effectively decreases the depressor response to isopropyl-norepinephrine.

Two other explanations of the decrease in responses are (1) chlorothiazide acts directly upon peripheral vascular smooth muscle inducing some refractoriness to agents tending to alter its state of contraction, or (2) the compound sensitizes the carotid sinus buffer mechanism and increases its effectiveness in opposing pressure changes. The reported reduction in dosage of ganglionic blocking drugs necessary for blood pressure reduction in hypertensive patients receiving chlorothiazide

(5) would support the first possibility.

Summary. Chlorothiazide (Diuril), an orally effective diuretic, has been shown to decrease arterial pressure response to injections of norepinephrine, epinephrine and isopropyl-norepinephrine in the anesthetized dog. The possible relationship of this finding to the reported hypotensive action of the compound is discussed.

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Indirect Staining Reaction with Fluorescent Antibody for Detection of Antibodies to Pathogenic Fungi. (23965)

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Methods for studying fluorescent antibody-antigen reactions have been widely used since their introduction by Coons *et al.* (1,2). They have made possible more definitive studies on the site of antibody formation in animals (3), on the distribution in tissues of polysaccharide (2), bacterial (4), viral (5) and Forssman antigen (6), and on specific localization of globulin in tissue from diseases believed to have hypersensitivity as an underlying cause (7). Recently Watson and Coons, as mentioned in Weller's and Coons' report (8), and Gitlin (9) have demonstrated that a rabbit antibody to human gamma globulin can be conjugated with fluorescein-isocyanate for the purpose of detecting any specific antibody of human origin after combination with its homologous antigen. Weller and Coons (8) were able to detect viral inclusion bodies using a 2-stage technic (indirect method) in which tissue sus-

pected of containing varicella was treated with a convalescent serum and then fluorescein-labeled rabbit antihuman gamma globulin. This general technic has been used in the present work and describes the reaction of pathogenic fungi with human antisera and rabbit antihuman gamma globulin labeled with fluorescein. The possible usefulness of this technic is compared to standard serologic procedures. In at least one disease, cryptococcosis, preliminary evidence suggests that the indirect fluorescent-antibody technic used here may be superior to standard serologic procedures.

Materials and methods. Fungi studied. Stock cultures of *Histoplasma capsulatum*, *Candida albicans*, and *Blastomyces dermatitidis* were maintained on appropriate media in the yeast phase of growth. The strain of *Cryptococcus neoformans* was freshly isolated

from a case of cryptococcal meningitis; the patient's serum was used for serologic studies. Other human antisera were obtained from patients with systemic manifestations of mycotic infection. The complement fixation titers of the blastomyces and histoplasma human antisera were 1:256 and 1:128 respectively. The yeast cell agglutination titer of the candida serum was 1:256. *Preparation and fluorescein-labeling of rabbit antihuman gamma globulin serum.* White rabbits weighing approximately 5 lb. were immunized with alum precipitated(10) human gamma globulin. The rabbit sera were pooled and fractionated by Cohn's ethanol method as described by Deutsch(11) to obtain the globulin fraction. The globulin was conjugated with fluorescein isocyanate using Goldman and Carver's modification(12) of Coons' technic (1). The fluor was stored at -20°C . *Preparation and staining of slides.* The fungi, with the exception of *B. dermatitidis*, were killed with 1% phenolized-saline, washed several times with normal saline and adjusted to a No. 2 MacFarland nephelometer tube. *B. dermatitidis* was killed with pyridine according to the method of Fischer and Labzoffsky (13). A loopful of organisms was spread on a clean slide ranging in thickness from 1.2 to 1.28 mm and allowed to air dry. A drop of undiluted or diluted human sera was then placed over the dried smear. After standing for 30 minutes under a petri dish cover with moistened filter paper liner, the slide was washed gently with 10 ml of isotonic phosphate-buffered saline (pH 7.4). A drop of fluorescein-labeled rabbit antihuman globulin was then placed on the slide and allowed to act for 30 minutes at room temperature. The slide was then washed in the above buffer for 10 minutes. A drop of glycerol-buffer (9 parts glycerol to 1 part isotonic phosphate-buffered saline, pH 7.4) was placed over the stained smear and a cover slip applied. *Light source and microscope used.* A carbon arc with automatic electromagnetic feed operated at 12 amperes D.C. was used with a Leitz monocular microscope fitted with a standard darkfield condensor and Wratten 2A filter in the eyepiece. Details are given in articles by Coons(1) and Coffin(14). *Controls for spe-*

cific staining. The autofluorescence of organisms was studied in unstained preparations and with the fluorescein-labelled rabbit antihuman globulin (Rabbit-fluor). To test the specificity of the positive reactions obtained when human antiserum and Rabbit-fluor were used together various absorptions of human sera with homologous and heterologous organisms were performed.

Results. C. albicans. Unstained *C. albicans* yeast cells killed with phenol, gave a dull bluish-green autofluorescence under the fluorescent microscope; with the Rabbit-fluor alone, they were somewhat greener. With human antiserum plus the Rabbit-fluor, a bright greenish-yellow fluorescent "ringing" of the individual cells was noted (Fig. 1). The term ringing best describes the bright staining reaction which appears to involve all or part of the cell wall structure. Ringing was present at serum dilutions comparable to those causing agglutination of yeast cells in the standard serologic test. Absorption of the human antiserum with *C. albicans* yeast cells removed the ringing while absorption with *H. capsulatum* cells did not. Several normal sera caused ringing, in keeping with the known fact that antibodies to *C. albicans* can be demonstrated in a large percentage of the population.

C. neoformans. Unstained *C. neoformans* cells appeared bluish-gray under the fluorescent microscope and essentially the same when the Rabbit-fluor was added. Capsules were not visible. When serum from the patient with cryptococcosis was added plus the Rabbit-fluor, the capsules were visible and appeared as opaque fluorescent halos about the cells. The capsular reaction was noted with diminished intensity at a 1:2 dilution and was negative at a 1:4 dilution of serum. Fig. 2 and 3 show the positive and negative capsular reaction obtained with serum from the patient with cryptococcosis and a normal serum respectively. A serum from this patient 4 months later, when he was in remission as a result of treatment, gave only a slight capsular reaction. Several normal sera gave a slight capsular reaction, although far less than the specific human sera. It was found subsequently that absorption of these sera with *C.*

albicans removed the slight capsular reaction from normal serum but not from the patient's

serum.

H. capsulatum. Unstained *H. capsulatum*

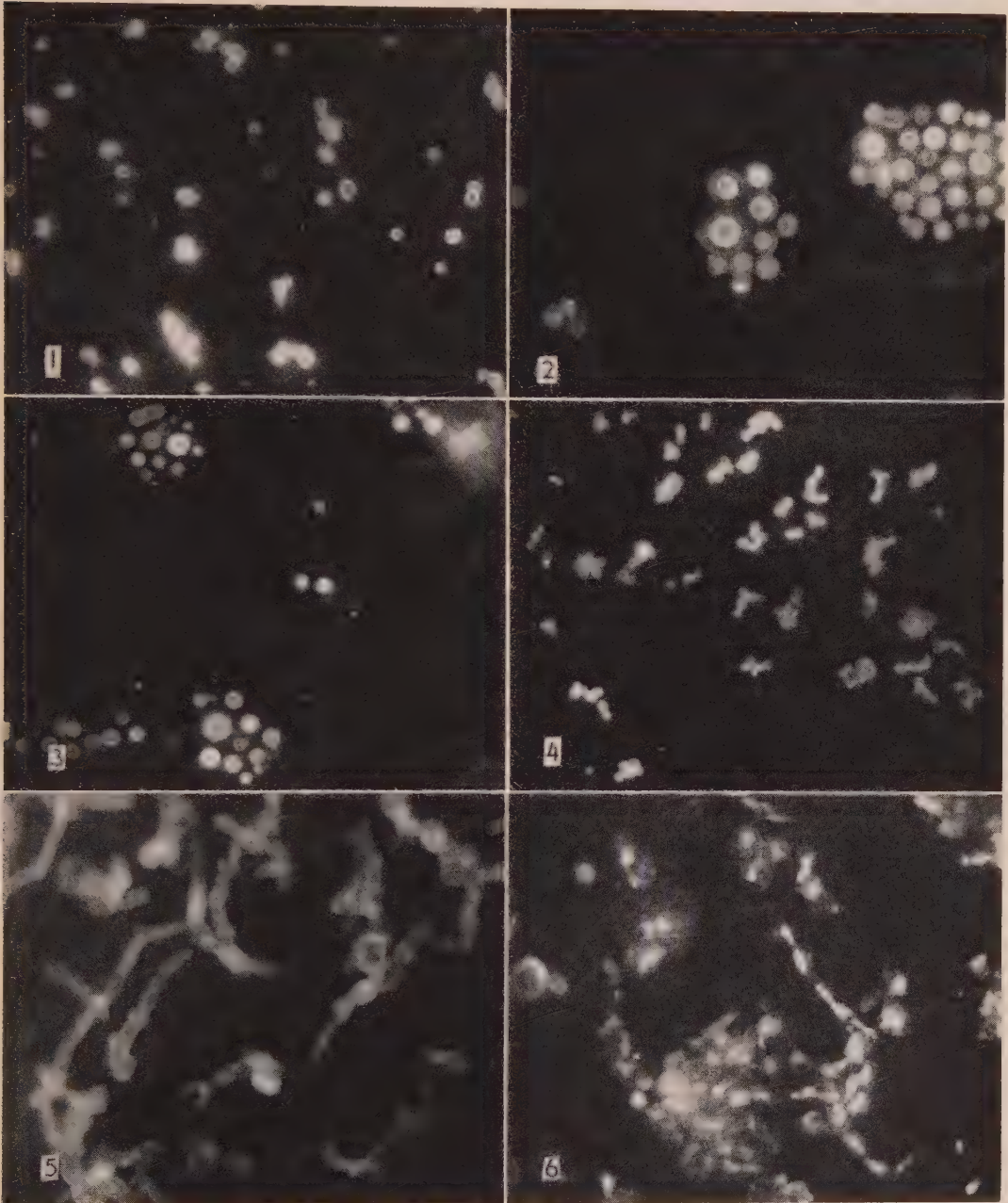


FIG. 1. *C. albicans* cells plus human antiserum and rabbit anti-human gamma globulin fluor. All figures 550 X.
 FIG. 2. *C. neoformans* cells plus human antiserum and rabbit anti-human γ globulin fluor.
 FIG. 3. *C. neoformans* cells plus normal human serum and rabbit anti-human γ globulin fluor.
 FIG. 4. *H. capsulatum* cells plus human antiserum and rabbit anti-human γ globulin fluor.
 FIG. 5. *B. dermatitidis* cells plus human antiserum and rabbit anti-human γ globulin fluor.
 FIG. 6. *B. dermatitidis* cells plus normal human serum and rabbit anti-human γ globulin fluor.

cells appeared dull green under the fluorescent microscope, and somewhat brighter when the Rabbit-fluor was used. With human antiserum and Rabbit-fluor, the cells took on a ringed, greenish fluorescent appearance (Fig. 4) much like the *Candida* cells mentioned above. Several normal sera were noted to give this effect also, despite negative complement fixation tests to *H. capsulatum* antigens. Absorption of the antiserum with Histoplasma cells abolished the ringing effect from all sera, however absorption with *C. albicans* or sheep red blood cells appeared to intensify the ringing effect in both normal and specific sera. The ringing effect, with specific human antiserum dilutions, persisted at approximately the same titer (1:128) as the complement fixation test while some normal human sera ringed cells in a dilution as high as 1:16. The reason for the activity of Histoplasma cells in normal sera is obscure at the present time.

B. dermatitidis. Unstained *B. dermatitidis* cells appeared bluish-green when observed under fluorescent illumination and somewhat greener when stained with the Rabbit-fluor alone. In Fig. 5 and 6 are shown *B. dermatitidis* cells in the yeast phase with some mycelial transformation, not infrequently observed in such cultures when a temperature of 37°C is not rigidly maintained. Fig. 5 shows the appearance of cells when a positive human Blastomyces serum and Rabbit-fluor are added. A bright greenish-yellow fluorescence which stains the cytoplasm diffusely is seen; this tends to make the cells appear larger than normal. The striking appearance of the positive reaction was seen through dilutions comparable to the complement fixation titer of the serum (1:256). Normal human serum and Rabbit-fluor failed to give this reaction; the fluorescence in this case was concentrated in the cytoplasmic inclusions of the cells (Fig. 6).

Reactions with fluorescein-labeled human gamma globulin. A sample of the immune human gamma globulin used to immunize rabbits was conjugated with fluorescein-isocyanate and used as an additional control. It failed to cause specific fluorescence with *H. capsulatum* or *B. dermatitidis* cells but did ring *C. albicans* cells brightly in what is considered a

positive reaction. This unusual result would indicate that gamma globulin (American Red Cross) contains a substantial amount of antibody reacting with *C. albicans*. A positive agglutination obtained with human gamma globulin and *C. albicans* yeast cells confirms the presence of such antibody in this material.

Discussion. In the present report all fungi examined in unstained preparations fluoresced with either a bluish-gray or light-greenish hue. It was impossible therefore to have a perfectly negative control composed of non-fluorescing organisms. It subsequently was found however, that when specific human serum and a fluorescein-labeled rabbit antibody to human gamma globulin were applied to the organisms, a characteristic fluorescing reaction took place with each fungus which clearly allowed the identification of specific antibody. These phenomena were fluorescent ringing of cells in the case of *C. albicans* and *H. capsulatum*, fluorescing capsular opacity with *C. neoformans*, and a diffusely even staining, bright fluorescence of hyphal elements in *B. dermatitidis*. The technic used here was primarily an attempt at the detection of antibody in sera from patients with suspected mycotic infections. In the case of blastomycosis, histoplasmosis, and candidiasis it would not seem to have any superiority over recognized serologic tests such as complement fixation or agglutination tests since the fluorescent titer in each case equalled the known serologic titer of the serum; however, in one disease, cryptococcosis, this technic may prove of value. Serum from a patient with cryptococcosis was tested unsuccessfully for presence of antibodies using the polysaccharide-coated resin particle test described by Evans(15), the capsular reaction test of Neill *et al.*(16) and the complement fixation test of Fischer and Labzoffsky(13). A positive test was obtained, however, with the fluorescent antibody technic suggesting that it is a more sensitive test.

Normal human sera were found to react with *H. capsulatum* cells in the fluorescent antibody technic described here; this activity could not be characterized as a cross reaction with either *C. albicans* or sheep red blood cells. It is possible that another antibody in

normal serum cross reacts with *H. capsulatum* cells or that a low titer of histoplasma antibodies is present and not detectable in the complement fixation test.

Summary. Antibodies in sera from patients with systemic mycoses were demonstrated by the indirect method of staining with fluorescent antibody. In spite of a general auto-fluorescence of pathogenic fungi, addition of human antiserum and a fluorescein-labeled rabbit antihuman gamma globulin resulted in characteristic fluorescing reactions. In the case of cryptococcosis antibodies were demonstrated by the fluorescent technic but not by other serologic methods.

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Comparison of Opacimetric and Hematocrit Methods in Measurement of Mitochondrial Swelling.* (23966)

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Study of the osmotic properties of isolated mitochondria is undeveloped in comparison with the maturity of similar studies of the red blood cell. The opacimetric method has been most widely used in mitochondrial studies for it is simple and lends itself readily to kinetic studies of volume changes. For the erythro-

cyte, the results obtained with the opacimetric method are generally in good agreement with the results of a variety of other methods which depend on different properties of the red cell system(1). For mitochondria, comparisons of the opacimetric method with others have been reported for only 2 alternative technics. Tedeschi and Harris(2) have shown that the optical density of mitochondrial suspensions is inversely proportional to the mitochondrial volume measured by photomicrography. The per cent dry weight of mitochondrial pellets

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[†] U.S.P.H.S. (Postdoctorate) Research Fellow of Nat. Cancer Inst.

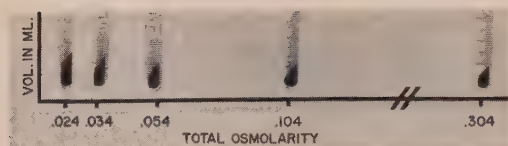


FIG. 1. Effect of osmolarity of medium on mitochondrial volume. Equal amounts of mitochondria sedimented in Shevky-Stafford tubes. Smallest division on tubes is .01 ml.

has been shown to decrease with decrease in optical density of the suspensions from which they are spun down(3). The latter correlation has been confirmed in this laboratory.

In the course of a study of mitochondrial swelling it was observed that mitochondrial pellets from suspensions of low optical density are macroscopically larger than those from more opaque suspensions of equal quantities of mitochondria. In this report quantitative data are presented which show that the volume of a mitochondrial pellet, *i.e.*, the mitocrit,[†] is inversely related to optical density of the suspension before centrifugation. These data compare the opacimetric and hematocrit methods for measuring relative mitochondrial volume.

Materials and methods. Male rats (Holtzman Co., Madison, Wis.) were fed *ad libitum*. Mitochondria were isolated(5) from a 13.5% homogenate of four livers in 0.30 M sucrose which was 0.002 M with respect to ethylenediamine tetraacetic acid (EDTA) at pH 7.4, by centrifugation for 12 minutes at 7100 x gravity (American Instrument Co., high speed refrigerated centrifuge, tube angle of 32°). They were washed twice in the same medium. The resultant stock suspension contained mitochondria from 1.5 g wet weight of liver per ml of sucrose-EDTA solution. Swelling was produced by diluting 2.0 ml aliquots of the stock suspension to 30 ml with a series of hypotonic sucrose solutions. Final concentrations in the series of mitochondria-sucrose systems were: 10% mitochondria, 0.002 M Tris (hydroxymethyl) aminomethane (Tris) buffer at pH 7.4, and sucrose at various molarities. For direct measurement of mitochon-

drial pellet volumes, 6.5 ml aliquots from members of this series were delivered to Shevky-Stafford graduated centrifuge tubes and centrifuged for 30 minutes at 5700 x gravity. The mean of the upper and lower limits of the top of the mitochondrial pellet was taken as its volume. For optical density measurements, 1.0 ml aliquots from the mitochondria-sucrose systems, at various sucrose molarities, were periodically diluted to 4.0 ml with sucrose solutions of a corresponding molarity, also buffered with 0.002 M Tris at pH 7.4. Optical density readings at 520 mμ were obtained with the Coleman Junior Spectrophotometer, Model 6A. After about 10 minutes at room temperature, slow secondary changes of the initial optical density values are observed. These secondary degenerative changes, also observed by Tedeschi and Harris(2), are believed to be similar to those occurring in CCl₄- or inorganic phosphate-induced isotonic swelling(5). It was found that during the time necessary for the mitocrit determinations (45-60 min), the optical densities of the corresponding mitochondrial suspensions could be maintained to within 4% of their initial values by keeping the mitochondria at 0-1°C, except during the short interval necessary for optical density readings which were carried out at 3-8°C.

Results. Fig. 1 is a photograph of mitochondria sedimented in Shevky-Stafford tubes. Each tube contained the same amount of mitochondria. It is evident that as the osmolarity of the medium decreases, the volume of the mitochondrial pellet increases.

The results from a repetition of this experi-

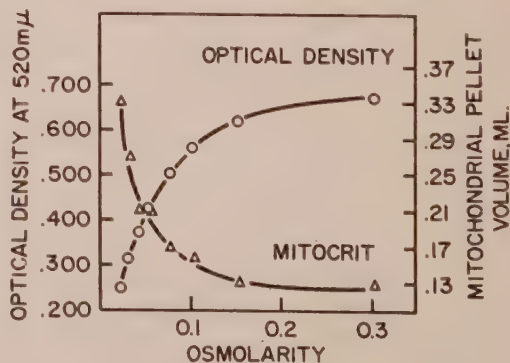


FIG. 2. Volume and optical density of mitochondria as functions of osmolarity of medium.

[†] The hematocrit, etymologically, refers to a blood measurement. Jackson and Pace(4) introduced the term mitocrit for measurement of mitochondrial pellet volume.

ment are shown in Fig. 2. Here, the mitochondrial pellet volumes and the corresponding optical densities have been plotted as functions of the final total osmolarity of the medium. The data clearly show that mitochondrial pellet volume increases continuously with progressive decrease in the osmolarity of the mitochondrial medium, and that associated with the volume increase there is a corresponding decrease in optical density of the mitochondrial suspension. This experiment has been performed, with minor variations, 5 times, always with the same result: mitochondrial volume increases as optical density decreases. The same result was also obtained in an experiment in which mitochondria in 0.30 M sucrose were induced to swell by the presence of inorganic phosphate.

For comparison of the rate of change of optical density with that of mitochondrial pellet volume, in Fig. 2 the units of the ordinates for the 2 parameters have been adjusted to make the total change in optical density equal to that in volume. The 2 curves are nearly symmetrical. However, as osmolarity decreases, the increase in mitochondrial volume at low osmolarities is somewhat greater than the corresponding decrease in optical density. This is probably due to incomplete packing of the grossly swollen mitochondria concomitant with their decreased specific gravity.

Discussion. A practical and a theoretical conclusion may be drawn from the results here presented. As to the former, measurements of mitochondrial pellet volumes confirm the validity of the optical density method of measuring relative mitochondrial volume. These data may be added to the evidence from photomicrographic measurements(2) and from measurements of mitochondrial water content(3). Thus, there is ample justification for choosing the simple opacimetric method for routine work.

Theoretically, the data are pertinent to the recently disputed view that mitochondria behave as osmometers. It is widely held that isolated mitochondria are impermeable to sucrose and similar non-electrolytes, and that they respond to osmotic changes in their environment according to classical osmotic pressure theory. Evidence for this view includes

the stability of mitochondrial volume in sucrose at or near 0.3 M(2,5,6), mitochondrial retention of a variety of cofactors, substrates, and ions through the isolation procedure(7), similarity in the rules governing transfer of water and non-electrolytes across mitochondrial membranes to those which apply to plant cell membranes and to the red blood cell(2), relative impermeability of mitochondria to sucrose shown by Jackson and Pace(4), and the studies of "colloid-osmotic swelling" in this laboratory(5) which indicate that mitochondrial membranes are impermeable to sucrose.

On the other hand, Werkheiser and Bartley (8) have presented data in support of the view that mitochondrial membranes are partially permeable to sucrose and possibly also to molecules as large as inulin. In addition, the view that mitochondrial volume changes are in accord with classical osmotic pressure theory has been challenged indirectly by Fomesu and Davies(9), on the grounds that small concentrations of adenosine monophosphate, with Mn^{++} or Mg^{++} , could largely protect the mitochondria from swelling in hypotonic sucrose solutions as low as 0.04 M. Possible alternative interpretations of these experiments are presented elsewhere(5). The results here reported on the behavior of mitochondria in hypotonic sucrose solutions clearly follow classical osmotic pressure theory. The progressive increase in mitocrit values as the external osmotic pressure is progressively decreased, is the expected behavior if the mitochondria are osmotic systems impermeable to sucrose. It may be pointed out that the permeability characteristics of the mitochondrial membrane can only be inferred indirectly from measurement of mitochondrial volume changes, regardless of the method used. Nevertheless, if future work confirms the recently expressed view that the mitochondrial membrane is permeable to sucrose (8), or that the mitochondria under certain circumstances do not adjust osmotically to external 0.04 M sucrose(9), then it would appear to us that a wholly new theoretical approach, other than classical osmotic pressure theory, will have to be taken to explain the experimental results presented here and in the

work of Tedeschi and Harris(2) and others, cited above.

Summary. The opacimetric and hematocrit methods for measuring relative mitochondrial volume are compared. Data from mitochondria swollen by exposure to hypotonic sucrose solutions demonstrate the validity of the simple opacimetric method, since, as osmolarity decreases, rate of increase in mitochondrial pellet volume approximates rate of decrease in optical density of the mitochondrial suspension. The results are discussed in terms of a current dispute regarding permeability of the mitochondrial membrane to sucrose.

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Action of Colicines on *Shigella boydii* and on their Rough and Mucoid Variants. (23967)

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Certain antibiotic substances produced by strains of *Escherichia* and *Shigella* have been defined as colicines. A recent review of colicines(1) has been published by Fredericq, a pioneer in this particular field. Halbert and Gravatt(2) have reported on the relation of *Shigella* type specificity and susceptibility to antibiotic producing strains of *Escherichia coli*. As extension and partial confirmation of their report, 170 *Shigella boydii* cultures[†] consisting of serotypes 1 through 11 and the 5 provisional serotypes were tested for possible characteristic patterns of sensitivity to 28 colicines. Some correlation between serological type and sensitivity to colicines has been revealed by this study.

The present report deals with an observation made during the testing of the aforementioned *Shigella* cultures. It was noted that

R (rough) and M (mucoid) colony variants seemed somewhat more susceptible to colicines than S (smooth) colonies of the same original culture.

Procedures. Further experiments were performed to establish the validity of these sensitivity differences. A group of colicine producing strains of *Escherichia* and *Shigella*[‡] were tested against randomly selected *Sh. boydii* strains utilizing the chloroform-killed plate assay technic of Gratia and Fredericq(3). R variants were obtained from an S colony of *Sh. boydii* 1, Strain No. 1701, by the methods indicated in Table I, and simultaneously tested with the S parent organism against colicines. The results shown in Table I and Fig. 1 indicate the marked sensitivity of R variants to some colicines employed in contrast to the relative resistance of the S parent culture. Sensitivity differences between R or M variants and their corresponding S parent cultures were also demonstrated in 2 other

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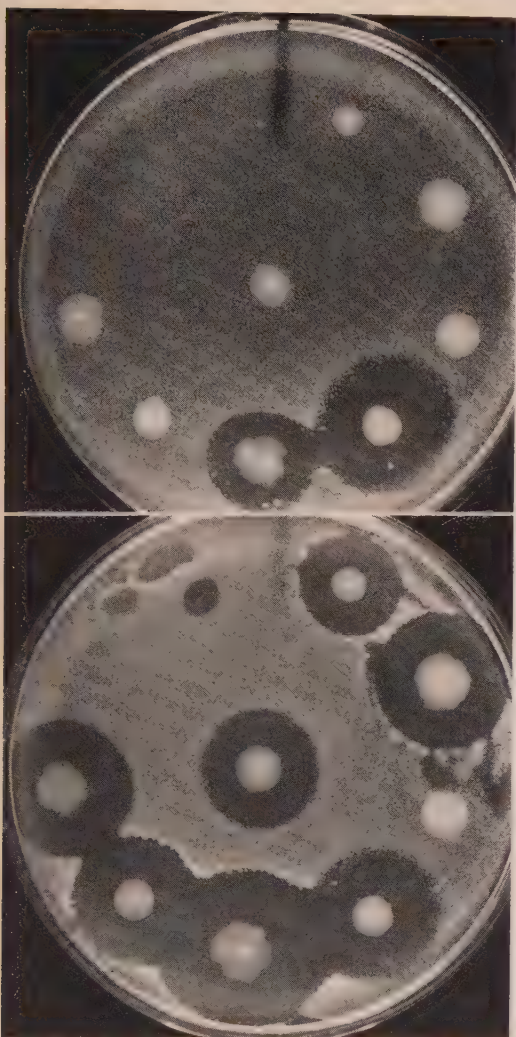


FIG. 1 (top). Colicine sensitivity of *Sh. boydii* 1, S parent culture.

FIG. 2 (bottom). Colicine sensitivity of *Sh. boydii* 1, R variant of culture in Fig. 1. Clockwise from top of plates, colicine producers are shown in the same order as listed in Table I.

TABLE I. Colicine Sensitivity of S and Non-S Variants of *Shigella boydii* 1, Strain No. 1701.

Colicine	S parent	Rough variants by		
		Prolonged broth incubation	.2% phenol broth	Homologous antiserum, 1:5 in broth
S2	0*	6	6	7
L	0	7	7	7
H	0	0	0	0
C	6	4	5	8
F	5	7	6	8
E	0	6	1	7
D	0	7	7	8
B	0	5	5	5

* Zones of inhibition measured in mm.

Results. S and R cultures of one strain of *Sh. boydii* 2 (Strain 1810) were tested for susceptibility to carbomycin, chloramphenicol, chlortetracycline, dihydrostreptomycin, erythromycin, furadantin, gantrisin, neomycin, novobiocin, oleandomycin, oxytetracycline, penicillin, polymyxin B, and tetracycline. There were no differences in sensitivity between the S and R cultures. However, differences between S and R variants of other bacterial species in regard to their sensitivity to a variety of antibiotics and bacteriophages have been reported in other studies(5-8).

S (smooth) pathogenic bacteria generally undergo population changes involving establishment of spontaneously arising non-S (avirulent) variants under usual conditions of laboratory cultivation. Braun and his co-workers(9) recently reported *in vitro* non-S to S population changes in *Brucella*, pneumococci and typhoid bacilli, brought about under the influence of bacterial desoxyribonucleic acid (DNA) plus desoxyribonuclease (D-Nase), or 6-furfurylaminopurine (kinetin).§ Some of the M variants of *Sh. boydii* 5 isolated in our study were incubated in broth containing kinetin in concentrations of 10^{-3} and 10^{-4} $\mu\text{g/ml}$. Within 4 days S organisms were obtained which satisfied all the usual criteria for S cultures, although type specific anti-S serum titers were somewhat lower than those found in the original S parent cultures. Colicines were tested simultaneously against the S parent organism, the M variants and the

§ We are indebted to Dr. A. F. Langlykke and Miss B. Stearns of E. R. Squibb and Sons for a sample of kinetin.

strains of *Sh. boydii* 1 and in cultures of *Sh. boydii* types 2, 4, 5, 7 and provisional serotype 703. Some colicines were inactive against both S parent and their non-S variants, other colicines exhibited similar antagonism against S parent and variants, but in no instance was the S parent culture significantly more sensitive to a colicine than its variant progeny. The generally accepted criteria for R and M organisms(4,5), were satisfied by all variants utilized.

TABLE II. Colicine Sensitivity of S, Induced M, and "Transformed" S Organisms of *Shigella boydii* Type 5, No. 2132.

Colicine	S parent		Induced M variants				Kinetin-induced S variants			
			M1		M2		M1-Tr		M2-Tr	
	S1	S2	1	2	1	2	1	2	1	2
534	0*	0	9	10	10	6	6	7	5	4
V	0	0	10	10	10	8	3	3	2	1
CF1	0	0	7	0	12	10	5	3	1	5
K	0	0	7	9	9	12	2	2	1	2
67	1	1	11	10	10	10	5	7	5	4

* Zones of inhibition measured in mm.

S organism obtained from the kinetin-passed M variants. The test plates showed a sensitivity gradation in which the S parent organism displayed relative resistance, the kinetin-passed S organisms were distinctly more susceptible and the M variants were markedly antagonized by the same colicines (Table II).

Discussion. The protective function of smooth surface somatic antigen of *Shigella* seems to be the most obvious explanation for the difference in action of colicines against organisms with and without this antigen. Gradual loss of surface somatic antigen by population change, and gradual recovery of this antigen in variant cells whose establishment was promoted by kinetin, results in corresponding colicine sensitivity changes. The validity of any possible characteristic colicine sensitivity spectrum for a certain *Shigella* serotype, therefore, may be regarded as partially dependent upon the protective effect exerted

by surface somatic antigen. The amount of this antigen present may account for some of the sensitivity fluctuations observed between cultures of identical serological composition.

Summary. In 58 experiments involving comparisons of 6 smooth parent cultures of various *Sh. boydii* serotypes and their corresponding non-S variants, the variants were significantly more sensitive to colicine action than the smooth parent strains. Reversal of the culture from the non-S to the S form was accomplished by use of kinetin, and the "transformed" cultures reflected, in part, the colicine spectrum of the original S parent culture and in part the spectrum of the non-S variants from which the "transformed" S cultures were immediately derived.

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Effects of Folic Acid Antagonist Therapy on Urinary Excretion of Formic Acid by Humans.* (23968)

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Although formic acid has been reported to occur in urine of normal human subjects on a mixed diet, the methods used for its determination have involved either extraction of

urine with ether and subsequent distillation of volatile acids from acid solution(1,2), or direct distillation of the urine(3-6). Significance of values obtained using these methods is somewhat doubtful since many naturally-occurring substances are known to yield formic acid upon treatment with acid at elevated

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TABLE I. Formic Acid Excretion in Subjects without Leukemia.

Subject	Sex	Age	Clinical data	Urinary formic acid, μ moles/hr
<i>Adults</i>				
60	♂	32	Normal control	16.8, 13.8
61	♂	35	<i>Idem</i>	23.4, 13.8
62	♂	29	"	16.3, 19.8
82	♂	65	Arteriosclerotic	32.0
83	♂	65	Metastatic carcinoma	10.4
84	♂	60	? Neurologic disease	20.5
85	♂	59	Angina pectoris	12.3
86	♂	67	Emphysema	14.7
87	♂	19	Idiopathic thrombocytopenia	21.5
88	♂	68	Pneumonia	47.3
91	♀	72	Aortic stenosis, angina pectoris	8.5
92	♀	68	Acute myocardial infarct	15.0
93	♀	64	<i>Idem</i>	13.2
94	♀	64	Congestive heart failure	9.9
95	♀	70	Angiocystic hamartoma	9.0
96	♀	60	Acute myocardial infarct	3.6
<i>Children</i>				
2	♂	12	Ulcerative colitis	1.9
5	♂	10	Streptococcal pharyngitis	8.5
8	♀	10	Repair of uretero-pelvic junction	3.8
45	♂	11	Rhabdomyosarcoma	5.9
46	♂	2	Neuroblastoma	1.5
90	♀	10	Fracture of fibula	15.7

temperatures. In animal studies with C^{14} -labeled substrates, sources of urinary formate have been shown to include sarcosine(7), α -carbon of glycine, glycolate, and glyoxylate, and β -carbon of serine, acetone, and methio-

nine(8). A major fraction of formic acid presented to the mammal is further metabolized (9-11), but profound impairment in capacity to utilize formate has been demonstrated in folic acid-deficient rats(11,12). This undoubtedly accounts for the marked increase in urinary excretion of formate by rats on folic acid-deficient diet(13).

The work to be reported indicates that formic acid is present in detectable quantity in urine of all human subjects studied. The levels in adult urine are lower than previously reported values(1,5). In children with acute leukemia undergoing folic acid antagonist therapy, excretion of formic acid is markedly increased. This investigation has been made possible by recent development of a sensitive and specific enzymatic method for determination of formic acid(14).

Methods. Timed urine samples were obtained from 3 healthy adults and from 19 patients on medical and pediatric services of Beth Israel Hospital. In addition, specimens were obtained from 22 children undergoing therapy for acute leukemia at Children's Cancer Research Fcn. Sixteen of the latter group were receiving the antifolic acid drug, amethopterin (Methotrexate®; 4-amino-4-deoxy- N^{10} -methylpteroylglutamic acid) in amounts listed in Table III. Pertinent clinical data are summarized in Tables I-III. Urine samples were refrigerated immediately, and either analyzed for formic acid within 18 hours, or frozen within that time and kept at -10° until

TABLE II. Formic Acid Excretion in Children with Acute Leukemia not Receiving Amethopterin.

Subject	Sex	Age	Clinical data	Previous amethopterin and interval since last given	Urinary formic acid, μ moles/hr
21	♂	13	In remission on 50 mg of 6-mercaptopurine daily	Yes; 5 mo	7.8
23	♂	4	In relapse; receiving adrenal steroids, chloramphenicol, and novobiocin	" 6 mo	16.8
24	♂	6	In relapse; receiving intrav. ACTH, chloramphenicol, and novobiocin	" 5 mo	22.1
26	♂	3	No previous therapy, receiving adrenal steroids	No	6.1
40	♀	7	Amethopterin omitted 14 days previously because of severe toxicity (stomatitis); partial remission	Yes; 14 days	11.2
50	♂	1	No previous therapy	No	3.6
53	♀	2	<i>Idem</i>	"	2.8

TABLE III. Formic Acid Excretion in Children with Acute Leukemia Receiving Amethopterin.

Patient	Sex	Age	Remission	Daily dosage of amethopterin, mg	Duration of treatment	Urinary formic acid, μ moles/hr
26	♂	3	Yes	1.25	4 wk	10.4
					5 "	8.3
					12 "	119
27	♀	5	"	1.25	3 mo	45.8
28	♂	4	"	2.5	6 wk	26.8
					18 "	85.8
29	♀	4	"	1.25	11 mo	49.5
30	♂	4	No	1.25 (& prednisone)	1 yr	32.0
33	♂	8	Yes	2.5 "	4 wk	8.4
					10 "	6.7
					12 "	6.7
					15 "	36.4
					18 "	66.1
34	♂	3	Partial	1.25	3 mo	123
					3½ "	165
				Amethopterin stopped 3 wk previously		9.7
				"	" 4 "	9.6
35	♂	3	Yes	2.5	6 mo	64
					8 "	117
					9 "	117
37	♀	7	No	2.5 (& prednisone)	2 "	14.0
41	♀	8	Yes	2.5 "	3 "	122
43	♂	9	"	2.5 "	3 wk	12.8
					12 "	72.1
					16 "	125
44	♀	5	"	1.25	6 "	10.5
					15 "	36.6
					22 "	265
47	♂	3	Early relapse	2.5	5 mo	60
					5½ "	204
				Amethopterin stopped 2 wk previously		72
				"	" 3 "	23.8
55	♂	3	In relapse	2.5	8 mo	301
				Amethopterin stopped 3 wk previously		10.5
56	♂	6	Incomplete (improving)	2.5	14 days	34.8
57	♂	10	Yes	2.5 & 5.0 on alternate days	1 mo	193

analyses were performed. Urines were brought to pH 7.0 with 5.0 *N* KOH, and assayed for formic acid according to the method of Rabinowitz and Pricer (14).

Results. Urinary excretion of formic acid by adults ranged from 3.6 to 47.3 μ moles/hour (Table I). Nonleukemic children and leukemic patients not receiving amethopterin excreted 1.5-22.1 μ moles/hour (Tables I and II). The 3 children with acute leukemia who had never received the folic acid antagonist excreted 2.8-6.1 μ moles/hour. Fifteen of 16 children receiving amethopterin excreted more than 32 μ moles/hour, and 9 patients excreted

over 117 μ moles/hour (Table III). An increase in formic acid excretion was usually not apparent until after several weeks of amethopterin therapy, and often not until long after remission of the leukemic process. Thus, in subject #33 formic acid excretion was only 6.7 μ moles/hour 12 weeks following start of treatment, but had increased by 10-fold after 18 weeks. A similar phenomenon was observed in patients 26, 43, and 44. This latent period may explain the low excretion of formic acid in patient 37, who had received amethopterin for 2 months. The level of urinary formic acid in treated children bore no apparent

relationship to clinical status of leukemia. Cessation of amethopterin administration to patients 34, 47, and 55 was followed by a sharp fall in formic acid excretion.

No evidence was found in urine of amethopterin-treated patients of substances that interfered with the formic acid assay. This was demonstrated by recovery experiments in which urine samples were reassayed after addition of known increments of formic acid; 100% recovery was obtained in all samples so tested.

Discussion. Our data demonstrate suitability of the enzymatic procedure of Rabinowitz and Pricer(14) for determination of urinary formic acid. Our studies indicate that in normal human subjects and in patients with a variety of disease states, formate may be detected in the urine, although in smaller quantity than previously reported(1,5). The variation in formic acid excretion from one patient to another, as well as in the same patient may be attributable to variations in dietary intake of formate precursors.

Participation of formic acid in metabolic processes is known to require a folic acid derivative(15-18). Formation of this derivative has been shown to be inhibited in animals receiving folic acid antagonists(19). Hence, formic acid would be expected to accumulate in folic acid deficiency and in subjects receiving folic acid antagonist therapy. This explanation undoubtedly accounts for the formaturia reported in folic acid-deficient rats(13) and for that observed in our patients treated with folic acid antagonist, amethopterin. Increased excretion of formic acid was often not seen until after several weeks or months of therapy.

It is of considerable interest that in several patients a complete remission had taken place well in advance of an increase in formic acid excretion, or in formiminoglutamic acid excretion(20). Since animal studies indicate that increase in urinary formic and formiminoglutamic acids is a reflection of folic acid deficiency in the whole organism(13), our observations provide *in vivo* evidence for the

thesis that leukemic cells are abnormally sensitive to folic acid deprivation(21).

Summary. An enzymatic procedure has been employed to demonstrate the presence of formic acid in small quantity in urine of normal human subjects and of patients with a variety of disease states. The excretion is markedly elevated in patients with acute leukemia receiving the folic acid antagonist, amethopterin.

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Radioactive Oxytetracycline (Terramycin*). III. Effect of Glucosamine HCl on Serum Concentrations.† (23969)

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Biological labelling of oxytetracycline by 2-C^{14} acetic acid has been reported(1). The need for such material to determine physiological handling and distribution had been felt for some time. Preliminary studies on its distribution in mice have been reported(2). One of the important questions that such a material might help resolve was the question of antibiotic blood levels—a problem difficult to attack with certainty by methods depending solely upon microbiological assay. The multiplicity of factors which might interfere with bioassay was realized, but no satisfactory method for eliminating or neutralizing them has been devised. Such factors include subtle interferences with the test due to antagonistic effects, chelation, serum protein binding, and adsorption by cellular elements of the blood. Extraction technics are always open to some doubt, especially with regard to completeness of extraction and dangers of biological inactivation of the antibiotic during the extraction process. Furthermore, quantitative extraction of blood can offer difficulties at the level of blood concentrations shown by most antibiotics. Hence, the importance of an independent assay method, such as isotopically labelled antibiotics afford, was not overlooked. Use of radioactive antibiotics in estimation of blood level can offer data which resolves many problems encountered in biological assay. Its precision and freedom from interferences of the kind that plague bioassay are great advantages. However, there are problems, too, especially those of relation between bioactivity and radioactivity at various time periods. Some of these will be discussed below.

Materials and procedures. Radioactive C^{14} oxytetracycline. The free base used contained

263 d/m/ μg . For administration to mice and dogs, it was dissolved in 0.01 N HCl. Sufficient 0.1 N HCl was added to produce complete solution. *Additives.* Glucosamine HCl tested for effects on serum level was dissolved with the antibiotic prior to administration. *Dosage.* Dogs were administered 1-2 ml of the radioactive solution by stomach tube at a dosage level of 10 mg/kg. Dosage in mice (administered by blunt hypodermic needle to the stomach) was 250 mg/kg. Male beagles weighing 8 to 13 kg each were used. The mice were inbred (brother-sister matings) Strain A produced in our laboratory from stock obtained from Jackson Memorial Laboratory. Male animals weighing 18-20 g were used. *Procedure.* 0.5 ml or more of whole blood was centrifuged; a measured volume of serum was removed into weighed 10 ml ampoules, and freeze-dried overnight at 200 μ . The ampoule was weighed, and the mg/ml solids were calculated by difference. Five to 10 mg of the solids were then weighed and evenly suspended in vials containing 20 ml of a homogeneous suspension of Thixcin (2.5%) in a toluene solution of 0.25% 2,5-diphenyloxazole(5), the background of which had been previously determined by liquid scintillation counting in a Tri-Carb Scintillation Spectrometer. After 4-6 ten minute counts had been taken on each sample, 200 μl of a toluene solution of standard benzoic acid containing 2674 d/m were added to each vial, and the counting efficiency of the vial and contents determined. The net counts on the sample were then evaluated by subtracting background; the net count was corrected to net d/m by application of the individual efficiency factor. This value was converted to d/m/mg by dividing d/m by the weight of sample counted, and converted to d/m/ml by multiplying by number of mg/cc of serum. Dividing this value by specific activity of the radioactive oxytetracycline produced a calculated value for the $\mu\text{g/ml}$ shown in the Tables.

* Registered Trademark, Chas. Pfizer & Co., Brooklyn, N. Y.

† We wish to acknowledge the cooperation of Dr. A. R. English and Miss Sophia Szendy, and the interest of Dr. B. A. Sobin, Dr. E. M. Weber, and Dr. J. H. Kane in this work.

TABLE I. Effect of Glucosamine HCl on Serum Levels of C¹⁴-Oxytetracycline.

Formulation	Dog	Time (hr)			
		0	.5	1	24
C ¹⁴ -oxytetracycline HCl	1	.0	.381	.780	.378
	2	.0	.309	.701	.427
	3	.0	.568	.353	.231
	4	.0	.618	4.48	.225
	Avg	.00	.469	1.58	.315
	<i>Idem</i> + glucosamine HCl (1:1)	6	.0	1.80	.978
8		.0	.611	4.03	.755
9		.0	1.06	8.32	.734
10		.0	.878	1.13	1.05
Avg		.00	1.087	3.61	.754
Ratio 2/1 (avg)	—	2.32	2.24	2.39	

Results. Table I presents results from an experiment designed to determine the effect of glucosamine HCl on rate of serum level increase and time of retention of radioactivity. It is apparent from the ratios of the averages that glucosamine HCl produced higher serum levels within one hour, and that these higher levels persisted at 24 hours (oxytetracycline or degradation products).

Table II indicates the data of a serum level study performed in inbred Strain A mice. These mice were chosen because of their excellent uniformity in other tests, *e.g.*, phagocytosis(6). These data indicate a serum level in favor of the glucosamine-oxytetracycline combination at every period of time assayed between 5 minutes and 24 hours following oral administration. These data also illustrate a trend which we believe to be significant in mice, *i.e.*, a decrease in serum levels between 15 minutes and 4 hours. This phenomenon is presently under detailed study.

Discussion. The radioactive oxytetracycline used in these studies is an example of a

compound which is labelled non-uniformly during its production from acetate precursor by *S. rimosus*(1). The preparation used here contained about 200 d/m/ μg . By increasing the size of sample that could be counted (from as little as fractional milligrams up to several hundred milligrams) with good efficiency (30-46%) through liquid scintillation counting, as little as 0.05 μg in as much as 5 cc of serum could be determined with good precision ($\pm 10\%$) within reasonable lengths of time. The excellent stability characteristics of the Tri-Carb Liquid Scintillation Spectrometer could be extended even further than was attempted here.

One of the advantages of the non-uniformly labelled C¹⁴-oxytetracycline over 7-tritio tetracycline, for example, is that one stands a better chance of picking up more biologically inactive metabolites of the antibiotic(3). Since the compound is labelled in the nucleus with carbon, it is not subject to losing its label by peripheral attack, such as a simple dehydrogenation of a tritium-labelled compound might accomplish.

One of the striking results of this study was to emphasize once again the tremendous individual differences with regard to serum levels shown by different individual animals (Table I). Since the radiochemical test is subject to greater precision than the microbiological test, one can now say more definitely that there are differences of this magnitude in the sera. However, the magnitude of the differences may arise not only from differences in absorption rate, but also by differences in binding equilibria between serum

TABLE II. Effect of Glucosamine HCl on C¹⁴-Oxytetracycline Serum Level in Strain A Mice.*

Time	C ¹⁴ -oxytetracycline		C ¹⁴ -oxytetracycline HCl + glucosamine HCl (1:1)	
	d/m/ml	$\mu\text{g/ml}$	d/m/ml	$\mu\text{g/ml}$
5 min.	972	3.70	1475	5.60
15	933	3.55	2050	7.80
30	1195	4.55	1432	5.44
60	641	2.44	703	2.67
120	954	3.63	1005	3.82
240	885	3.36	1135	4.31
24 hr	421	1.56	563	2.14

* Each figure is avg of 2 mice.

and solid elements of the blood(2). It is suggested that the particular immunochemistry of an animal, insofar as it determines adsorption forces of the surfaces of cells and macromolecules, may be a factor in causing these differences. An *in vitro* and *in vivo* study of these phenomena with radioactive oxytetracycline and tetracycline is presently underway.

Comparisons have been made of the ratios of radioactive assay to microbiological assay. As might be expected, the radioactive assay has given uniformly higher calculated values in serum for oxytetracycline than the microbiological assay has indicated. The differences appear to be accounted for if one postulates inactive complexes of oxytetracycline with blood constituents (*e.g.*, amino acids, ions, proteins and protein fragments and cells) and/or rapid metabolic conversion to inactive forms by the liver or other tissues. Data in C-57 mice(2) and in Swiss mice with H³-tetracycline(4) have indicated that high liver concentrations of oxytetracycline are achieved early; it is most probable that the antibiotic undergoes metabolic changes, including both degradation and conjugation to inactive forms. The products of these changes then return to the blood stream without significant bioactivity.

With respect to the observed advantage in serum blood levels produced by glucosamine (Tables I and II), the following mechanisms are suggested as possibilities: 1. Increased

absorption. 2. Decreased elimination by bile, urine, or feces. 3. Alteration in the binding equilibria between cells, proteins, and aqueous phases of the blood. 4. Alteration in cellular permeability, in favor of extracellular spaces. 5. Alteration in liver metabolism, whereby less of the absorbed antibiotic is sequestered by this organ.

Summary. 1. A new technic for studying serum levels of oxytetracycline is presented, involving estimation of the C¹⁴-oxytetracycline and derivatives thereof in serum solids by liquid scintillation counting. 2. The technic is illustrated by data from studies on the effect of glucosamine HCl on serum levels of beagle dogs and Strain A mice. 3. Enhanced serum levels were found with glucosamine HCl between 0 and 24 hours in dogs and mice. Various hypotheses, which are currently being tested, were advanced to explain this phenomenon.

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Effect of Hydrocortisone on Activation and Activity of Anaphylatoxin *in vitro*. (23970)

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The physiological importance of anaphylatoxin, a naturally occurring substance in blood of rat, guinea pig, dog, cat, horse, cow and man, has not yet been established(1). Rocha e Silva and Aronson have shown that anaphylatoxin becomes a potent histamine releaser when activated by incubation with polysaccharides such as inulin, dextran, and

agar(2). The activation takes place rapidly when plasma is incubated with agar at 37°C; time and temperature curves have been determined by Rothschild and Rocha e Silva (3). Anaphylatoxin preparations produce contractions of isolated guinea pig ileum, attributable to histamine release because they are blocked by antihistaminic drugs(3). Fur-

TABLE I. Effect of Hydrocortisone on Activation of Anaphylatoxin *In Vitro*.

Cone. hydrocortisone	Inhibition
2.5 mg/ml	11/11
1.25	18/24
.25	0/2

thermore, as isolated ileum is treated repeatedly with anaphylatoxin, tachyphylaxis soon takes place believed to result from exhaustion of the store of bound histamine in muscle, since sensitivity of muscle to histamine remains unimpaired.

Mucopolysaccharides of the ground substance and synovial fluid have for some time been implicated (by association) with the development of inflammatory reactions. Activation of anaphylatoxin by polysaccharides *in vitro* has led Rocha e Silva to suspect mucopolysaccharides as potential activators *in vivo* of anaphylatoxin(1). To approach the problem of inflammatory reactions, it was of interest to study the effects of hydrocortisone on activation and activity of anaphylatoxin *in vitro*.

Methods. Blood was collected from Sprague-Dawley rats under light ether anesthesia by cardiac puncture using heparin, 0.1 mg/ml of blood, as anti-coagulant. Plasma anaphylatoxin was assayed *in vitro* on guinea pig terminal ileum. The muscle was suspended in 4 ml oxygenated Tyrode's solution at 30°C, and muscle contractions recorded on a kymograph. One minute elapsed after chamber was thoroughly washed with Tyrode's solution, before any new substance was introduced for test. **Exp. 1.** Separate aliquots of rat plasma were treated as follows: 1) diluted 1:1 with saline as control; 2) activated by mixing 1:1 with 0.1% agar solution; 3) activated with agar in presence of hydrocortisone-hemisuccinate or equivalent amount of neutralized succinic acid; 4) mixed with hydrocortisone-hemisuccinate or neutralized succinic acid, without agar, as another control. The mixtures were incubated 10 minutes at 37°C, then 0.01-0.1 ml were transferred to tissue bath and tested on isolated ileum. Samples to be compared were tested in the order A,B,C,D, A to ascertain whether a similar muscular response could be

obtained with A after exposure to the other substances. Allowing for any decrease in responsiveness of muscle, it was determined whether A,B,C, and D were comparable in activity. **Exp. 2.** Anaphylatoxin was activated with agar and assayed *in vitro* on guinea pig ileum as in Exp. 1. Hydrocortisone-hemisuccinate or equivalent neutralized succinic acid was introduced directly into tissue bath immediately before the activated plasma, to determine effects on activity of anaphylatoxin *in vitro*. Trials in a series were rotated as in Exp. 1 to allow for tachyphylaxis of the isolated ileum.

Results. **Exp. 1, effect of hydrocortisone on activation of anaphylatoxin in vitro.** Anaphylatoxin activated by agar in the presence of 2.5 mg/ml hydrocortisone (as the hemisuccinate) was only half as active as anaphylatoxin activated with agar alone. In 18 of 24 trials with 1.25 mg hydrocortisone/ml, activation of anaphylatoxin was inhibited (Table I). Activity of hydrocortisone-hemisuccinate was not attributable to the succinate since equivalent concentrations of succinate alone did not produce inhibition in Exp. 1 or 2.

Exp. 2. Effect of hydrocortisone on activity of anaphylatoxin in vitro. At 1.25 mg/ml in the tissue bath, hydrocortisone clearly inhibited response of ileum to activated anaphylatoxin (Table II). Responses of isolated ileum to exogenous histamine, acetylcholine, and bradykinin were also inhibited by 1.25 mg hydrocortisone/ml in the tissue bath (Table III). Inhibition of muscle response was only temporary since the muscle responded to these agents as soon as hydrocortisone was washed out of bath.

In another experiment blood levels of anaphylatoxin were studied. Pretreatment of rats with 10 mg hydrocortisone acetate 24 and 4 hours prior to bleeding did not decrease anaphylatoxin activity in 6 of 8 trials. Ana-

TABLE II. Effect of Hydrocortisone in Tissue Bath on Activity of Anaphylatoxin *In Vitro*.

Cone. hydrocortisone	Inhibition
1.25 mg/ml	7/7
.625	3/7
.25	0/3
.125	0/2

TABLE III. Effect of Hydrocortisone in Tissue Bath on Activity of Exogenous Histamine, Acetylcholine, and Bradykinin *In Vitro*.

Substance	Cone. hydrocortisone	Inhibition
Histamine	1.25 mg/ml	13/13
	.625	4/6
	.25	4/4
	.125	1/5
Acetylcholine	1.25	9/12
	.625	6/8
	.125	0/4
Bradykinin	1.25	7/7
	.625	7/7
	.125	0/6

phylatoxin levels in rats bearing pneumodermal pouch inflammations(4) for 7 to 10 days were the same as in normal animals, in 6 of 7 comparisons.

Discussion. Haining found that sodium salicylate did not inhibit activation of anaphylatoxin *in vitro*(5). Exp. 1 demonstrated, however, that hydrocortisone in the incubation medium inhibited activation of anaphylatoxin. The pH of Tyrode's solution was not changed by the hydrocortisone, and calculations showed that the ionic strength was increased less than 5%, which would not explain the inhibition, since Rothschild and Rocha e Silva have reported that increases in ionic strength of the order of 100% are required to inhibit activation of anaphylatoxin (3).

In the second experiment the mechanism by which hydrocortisone inhibited the response of the isolated muscle to anaphylatoxin was not established unequivocally. As hydrocortisone did not inhibit the tachyphylaxis produced by anaphylatoxin alone, histamine release was not blocked; therefore hydrocortisone must have inhibited the muscle response to liberated histamine. Since acetylcholine, bradykinin, and histamine were all inhibited by hydrocortisone, the most likely mechanism for hydrocortisone would appear to be a non-specific desensitization of the muscle rather than by blockade of histamine receptor sites. Lefcoe has reported that comparable concentrations of hydrocortisone inhibit the responses of guinea pig tracheal muscle to histamine and cat tracheal muscle to acetylcholine(6).

Haining demonstrated that 4 mg sodium salicylate/ml inhibited the action of anaphylatoxin on isolated guinea pig ileum(5). Sodium salicylate prevented histamine release, however, since repeated anaphylatoxin treatments in the presence of salicylate did not produce tachyphylaxis.

In our experiments, hydrocortisone inhibited both activation of anaphylatoxin *in vitro* and response of isolated muscle to it. Hydrocortisone probably does not influence the inflammatory process by affecting activation or activity of anaphylatoxin *in vivo* since concentrations of hydrocortisone that were active *in vitro* were at least 1000 times greater than the levels of 11-oxygenated steroids usually quoted for peripheral blood(7,8). Furthermore, neither inflammation in the pneumodermal pouch nor administration of hydrocortisone to normal rats changed circulating levels of anaphylatoxin. These considerations suggest that anaphylatoxin does not play an important role in inflammation. Local alterations in the mucopolysaccharides at the site of inflammation, which could not be simulated in these experiments, still might regulate the process of inflammation *via* anaphylatoxin; but the role of anaphylatoxin in the inflammatory process remains to be demonstrated.

Summary. Effects of hydrocortisone on activation of and response to anaphylatoxin were studied using isolated guinea pig ileum. Hydrocortisone at 1.25 mg/ml inhibited activation of anaphylatoxin *in vitro*. At the same concentration in the tissue bath it also inhibited effects of activated anaphylatoxin on isolated guinea pig ileum. This inhibition was attributed to a non-specific desensitization of the muscle rather than to direct inhibition of anaphylatoxin or histamine release.

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Effect of Low Sodium Diet on Sodium Content and Radiosodium Exchange in Rat Bone.* (23971)

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Skeletal tissues are rich in sodium, a part of which readily exchanges with radiosodium. In recent years a number of workers have been interested in studying the degree of lability of this skeletal component under a variety of experimental situations. Among these is the effect of a low sodium diet.

Orent-Keiles, Robinson and McCollum(1) noted that the bones of rats reared on a low Na diet were soft in consistency. Bergstrom and Wallace(2) found that the bones of young rats kept on a low Na diet for 2 weeks contained only half as much Na as the controls. More recently Munro, Satoskar and Wilson (3) in a study of adult rats observed a decrease of 10% in bone Na content during a 14 week period of low Na feeding. The latter workers also determined radiosodium exchangeability in the bones of their animals, and finding no change concluded that the Na lost from bone came from the non-exchangeable fraction.

The present study was undertaken to investigate this problem.

Experimental. Twelve 20-day-old male albino rats of the Rochester strain were fed Big Red dog pellets and tap water for 10 days, at which time they were divided into 2 equal groups according to a table of random numbers. The flip of a coin then decided which group was to receive the low Na diet. The diet was constructed by blending Crisco 300 g, Lonalac 600 g, rolled oats 2070 g, brewer's yeast 30 g, CaCl_2 12 g, CaHPO_4 •

$2\text{H}_2\text{O}$ 11.4 g, and polyvisol 1.8 cc. On analysis it was found to contain 0.011% Na, 0.7% Cl, 0.42% K and 0.44% Ca. For the control group 60 g of NaHCO_3 was added, bringing the Na concentration to 0.55%. Distilled water was provided for drinking.

Since preliminary trials had indicated that food consumption was poor on the low Na diet, it was decided to pair-feed the 2 groups. Records of food consumption were kept, and the control group offered only as much food as the average consumed by the low Na group.

The experimental period lasted 4 weeks. The animals were then given radiosodium²² ($2.5 \mu\text{c}/100 \text{ g}$) by intraperitoneal injection, and $2\frac{1}{2}$ to 4 hours later killed by decapitation. The long bones were dissected free of adherent muscle and periosteum, the ends clipped off, and the marrow removed. Bone water was determined by drying *in vacuo* at 70°C . The dried samples were then ashed at 525°C and the ash taken up in a small amount of 2 N HCl. Sodium content was determined in this ash solution by means of a Baird internal standard flame photometer equipped with a Na III-Li II filter and a manufactured gas burner, with natural gas as a fuel. Using this apparatus we found that interfering substances present in bone produce a small, and constant, error in sodium readings over a concentration range of 0.1-0.4 meq/l. Serum sodium was determined by flame photometry, and chloride by Volhard titration. Radiosodium was determined in a scintillation counter.

In view of the uncertainty regarding the position of Cl in bone, the total Na con-

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tent of bone was partitioned into 2 phases—*fluid* Na and *crystal* Na—on the basis of bone water content rather than the calculated Cl space(4). By this method of calculation, fluid Na = serum Na $\times$ bone H₂O $\times$ 0.942/0.93, and crystal Na = total Na – fluid Na, where 0.942 is the Donnan factor and 0.93 the assumed serum H₂O content. Radiosodium values were treated in a similar manner, and on the assumption that fluid Na of bone had the same specific activity† as serum, the specific activity of the crystal Na fraction could be calculated. Radiosodium exchangeability was then expressed as the ratio: S.A. bone crystal Na/S.A. serum Na.

Results. Table I lists body weights for the 2 groups of rats. The low Na group grew more slowly than controls, and by end of the third week had stopped growing altogether. Values for various measured constants in bone and serum are listed in Table II. There was a modest decrease in concentration of sodium in both serum and bone. This decrease involved both calculated fluid Na and crystal Na phases. Radiosodium exchangeability also decreased in response to the low Na diet and it should be noted that percentage decrease in exchangeability was equal to decrease in crystal Na content. Thus the observed change in

crystal Na involved only the exchangeable fraction, and there was no detectable change in the non-exchangeable fraction.

Discussion. It has long been known that a certain minimum intake of Na is necessary for normal growth in the rat(1). Evidently the degree of Na restriction in our experiments was sufficient to interfere with growth.

The principal question which these experiments were designed to answer was whether the newly forming bone in the young, rapidly growing animal would contain less than normal amounts of Na, and further to decide whether exchangeable or non-exchangeable fraction of bone crystal Na would be involved in this change.

The magnitude of the change in bone Na content was comparable to that observed by Munro *et al.*(3) in older rats fed a low Na diet, but distinctly less than that observed by Bergstrom and Wallace(2) in young rats. Changes in serum Na were comparable in all 3 investigations. In constructing their low Na diet the latter authors substituted NH₄⁺ ion for Na in the incorporated salt mixture, and perhaps this accounted for the larger decrement in bone Na in their animals.

From our data it would appear that the difference in bone Na content in the 2 groups of animals can be accounted for in the exchangeable fraction of crystal Na. This is quite in keeping with observations made in our laboratory on bone Na changes during diabetic acidosis, in which decrease in crystal Na was matched by a decrease in radiosodium exchangeability(5). Munro *et al.*(3) on the

TABLE I. Body Weight, g.

Age (days)	Control group	Low Na group
30	69 $\pm$ 13*	74 $\pm$ 10
50	135 $\pm$ 15	102 $\pm$ 14
58	150 $\pm$ 13	106 $\pm$ 13

* Stand. dev.

TABLE II. Serum and Bone Composition.

Constituent	Control group		Low Na group		Difference (%)	"P"
Serum Na, meq/l	145	$\pm$ 1 *	133	$\pm$ 2	- 8	
" Cl, "	101	$\pm$ 1	93	$\pm$ 2	- 8	
Bone H ₂ O, %	29.2	$\pm$ 2.2	27.4	$\pm$ 2.2	- 6	
" Cl, meq/g wet wt	.0205	$\pm$.0028	.0195	$\pm$.0028	- 5	
Total bone Na, meq/g wet wt	.168	$\pm$.005	.155	$\pm$.006	- 8	<.01
" " " dry "	.237	$\pm$.003	.214	$\pm$.006	-10	
Crystal Na, meq/g dry wt	.176	$\pm$.007	.163	$\pm$.007	- 7	"
S. A. crystal Na/S. A. serum Na	.49	$\pm$.04	.43	$\pm$.02	- 6	"
Exch. crystal Na, meq/g dry wt	.086		.070		-19	
Non-exch. " " " " "	.090		.093		+3	

* Stand. dev.

† counts/min./meq. Na.

other hand observed no change in radiosodium exchangeability in their animals, and reached a conclusion quite different from ours—namely that the decrement in bone Na was due to a change in the non-exchangeable fraction. Munro *et al.* used a 24-hour period for radiosodium equilibration whereas we employed a much shorter time.

On the basis of our data it would seem that the division of bone crystal Na into two phases—exchangeable and non-exchangeable—has a physiological justification. In the present experiments, and those of Lobeck and Forbes(5) on diabetic acidosis, it was shown that Na lost from bone came from the exchangeable fraction.

Summary. Young rats, reared on a low Na diet, manifested growth failure, and their

bones contained 8-10% (depending on method of calculation) less Na than pair fed controls. Radiosodium exchangeability declined by a similar amount, and it is concluded that the change in Na content involved the exchangeable fraction.

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Effect of Low Potassium Diet on Cyclopropane-Epinephrine Ventricular Tachycardia and Arterial Plasma Potassium in Dogs.* (23972)

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Meek *et al.*(1) observed that epinephrine in doses which cause only occasional ventricular extrasystoles in unanesthetized dogs produced ventricular tachycardia during cyclopropane anesthesia. O'Brien *et al.*(2) found that the onset of arrhythmia was associated with a rapid and marked rise in arterial plasma potassium. Since liver is the primary source of potassium mobilized by epinephrine (3,4), O'Brien *et al.*(5) eliminated the hepatic circulation surgically and found a smaller rise in potassium as well as protection from arrhythmia in 17 of 18 dogs.

This study was undertaken to seek a non-surgical method for reducing the rise in arterial plasma K that normally results from an epinephrine injection and to determine whether or not any K changes that might be found could be related to the occurrence of cyclopropane-epinephrine ventricular tachy-

cardia.

Methods. After administering cyclopropane to dogs for 30 minutes, 0.005 mg/kg epinephrine in 5 ml isotonic sodium chloride was injected intravenously at a constant rate of 1 ml per 10 seconds. Two control determinations of the occurrence and duration of ventricular tachycardia were made one week apart and with the second determination, the changes in arterial plasma potassium concentration were studied. Effects on cardiac rhythm were determined from electrocardiographic records. Blood was rapidly withdrawn from the femoral artery into heparinized syringes before and at intervals of 20, 40, 60, 80, 100, 120 and 150 seconds after the beginning of the epinephrine injection. Plasma was analyzed for potassium with the Beckman flame spectrophotometer(6). After control studies, 20 dogs were placed on a low potassium diet for 2 to 3 weeks following which the effects of epinephrine on arterial plasma potassium and cardiac rhythm were again noted.

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TABLE I. Effect of Low Potassium Diet on Cyclopropane-Epinephrine Ventricular Tachycardia and Arterial Plasma Potassium in Dogs.

Dog	Control				After 2-3 wk on diet			
	Before Epi.	After Epi.*			Before Epi.	After Epi.*		
	Plasma K conc. (mg %)	Max plasma K conc. (mg %)	Duration of VT (sec.)	% rise in K	Plasma K conc. (mg %)	Max plasma K conc. (mg %)	Duration of VT (sec.)	% rise in K
1	16.9	32.3	22	91	10.5	14.0	0	33
2	13.4	23.7	47	77	11.7	16.6	5	42
3	14.6	28.0	50	92	12.3	18.6	7	51
4	15.7	30.6	90	95	10.9	19.7	0	81
7	13.7	25.4	42	85	10.9	13.1	0	20
9	15.4	23.1	63	50	12.0	18.3	0	53
10	16.6	26.9	54	62	15.7	22.9	0	46
11	15.4	26.9	37	75	12.0	19.7	0	64
15	17.1	25.7	54	50	10.9	22.3	0	105
16	18.3	40.0	68	119	12.9	22.0	0	71
20	13.7	27.4	61	100	10.6	14.3	0	35
Avg	15.5	28.2		82	11.9	18.3		54
5	14.3	26.9	73	88	12.3	18.9	73	54
6	15.7	37.1	67	136	10.9	26.3	43	141
8	13.1	32.9	73	151	11.7	27.4	50	134
12	16.3	26.3	57	61	11.7	16.9	25	44
13	14.9	30.9	63	107	11.1	24.9	40	124
14	14.6	29.1	61	99	8.6	16.6	53	93
17	15.1	34.3	90	127	10.6	18.6	50	75
18	16.3	35.1	60	115	16.6	31.1	40	87
19	17.4	33.4	51	92	16.0	41.5	44	159
Avg	15.3	31.8		108	12.2	24.7		102

* Epi. = .005 mg/kg epinephrine.

VT = ventricular tachycardia.

Results. On the 2 control determinations for the occurrence and duration of ventricular tachycardia, arrhythmias were observed on both tests in all animals. In all but 2 dogs (No. 10 and 18) the resting level of plasma potassium was lowered by the low potassium diet and in all cases but No. 19, the maximum plasma K level following epinephrine injection was also reduced. Results arranged according to the effect of low potassium diet on cyclopropane-epinephrine ventricular tachycardia are shown in Table I. Eleven had a marked reduction or absence of cyclopropane-epinephrine ventricular tachycardia and the average increase in potassium above resting level in these animals after injection of epinephrine was only 6.4 mg% (or 54%) compared with 12.7 mg% (or 82%) on their control. Nine dogs continued to show irregularities when tested after 2 to 3 weeks on the low potassium diet. The average increase in potassium in these 9 dogs after injection of epinephrine was 12.5 mg% (or 102%) above their resting level compared to 16.5 mg% (or 108%) on their control.

Discussion. Eleven of the dogs on a low K diet were in some way protected from ventricular tachycardia after injection of epinephrine during cyclopropane anesthesia and 9 were not. The question is whether these results are related to the K changes in the respective groups. In the 11 protected dogs it may be noted that not one reached a maximum K level after injection of epinephrine equal to that obtained when it was used as a control and showed ventricular tachycardia. This group of 11 on control studies showed an average K increase of 82% after epinephrine injection while after being on a low potassium diet the same injection of epinephrine resulted in an average increase of only 54%. The data on this group indicate that an increased plasma K level could be of importance in sensitizing the heart to epinephrine during cyclopropane anesthesia.

Of the 9 dogs on the low K diet that continued to show ventricular tachycardia after injection of epinephrine, the plasma K level in 4 failed to rise above that of the protected group. Therefore, according to our thinking,

these should have been protected; although this was not the case there was considerable reduction in duration of ventricular tachycardia in 2 animals (No. 12 and 17). In the remaining 5 the maximum K level was significantly above that of the protected group when ventricular tachycardia occurred. Although the reason for these variations is not clear, the fact that ventricular tachycardia was seen in 5 cases when the maximum K level was near that of their respective controls is at least some evidence for an increase in cardiac irritability being associated with an increase in plasma K.

In recent studies Dresel and Nickerson(7) have noted that some "factor" brought to the heart by the venous blood is probably involved in epinephrine induced arrhythmias during pentobarbital anesthesia. However, they doubted that the rise in arterial plasma potassium caused the arrhythmias. There does, however, seem to be justification for the idea that at least during cyclopropane anesthesia, regardless of what other factors might be involved, the rapid increase in potassium may contribute in part to the production of arrhythmias since protection from these arrhythmias was afforded by elimination of hepatic circulation which also markedly reduced potassium mobilization(5). The present studies tend to confirm this idea with the further indication that the relative increase in potassium may also be of some importance. Whatever the "factor" referred to by Dresel and Nickerson might be, it is possible that it

may be associated with the release of potassium from the liver in a large proportion of animals.

Summary. The effect of a low potassium diet of 2 to 3 weeks duration on cyclopropane-epinephrine ventricular tachycardia and arterial plasma potassium has been studied in 20 dogs. Eleven dogs were protected from the arrhythmias while on the diet and 9 were not. In the protected group the K mobilized by epinephrine was considerably reduced. In the non-protected group the per cent rise in K was on the average approximately the same as in control. It is concluded that these studies confirm the view that an actual or relative increase in plasma potassium contributes, at least in part, to the increased cardiac sensitivity to epinephrine during cyclopropane anesthesia.

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Mediation of Progestational Action of Amphenone by the Rabbit Adrenal. (23973)

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Progestational proliferation of rabbit endometrium after parenteral treatment with amphenone* has been described(1). With the

exception of certain steroids of gonadal, adrenal or synthetic origin, amphenone is the only compound known to exert progestational activity in the rabbit. This unique action of a non-steroid raises the question whether amphenone exerts a direct effect on the rabbit

* The structural formula for amphenone according to recent revision(2,3) is 3,3-bis(p-aminophenyl)-2-butanone. We have used the dihydrochloride.

TABLE I. Progestational Response to Amphenone in the Rabbit following Alteration of Endocrine Status.

Series	No. of animals	Compound	Total dose (mg)	Operative procedure	Progestational proliferation
I	4	amphenone	3000.0	None	+4, +3, +4, +4
	7	"	2000.0	"	+2, +3, +3, +1, +2, +3, +3
	5	progesterone	.5	"	+4, +4, +4, +4, +4
	5	none		"	0, 0, 0, 0
II	6	amphenone	3000.0	Ad. + Ov.*	0, 0, 0, 0, 0, 0
	4	progesterone	.5	"	+4, +4, +4, +3
	8	amphenone	3000.0	Ov.	+4, +4, +3, +1, +3, +3, +2, +1
III	3	cortisone	27.5	None	0, 0, 0
	4	cortisone + amphenone	27.5 3000.0	"	0, 0, 0, 0
	3	cortisone + progesterone	27.5 .5	"	+3, +4, +4, +4
	3	cortisone + amphenone + progesterone	27.5 3000.0 .5	"	+3, +3, +3
	3	ACTH†	500.0	"	0, 0, 0
IV	3	progesterone	.5	"	+4, +4, +4

* Bilateral adrenalectomy plus bilateral ovariectomy.

† Armour's ACTHAR given daily subcut. in peanut oil with 5% beeswax. Dose in mg equivalents of standard LA-1-A.

uterus or whether its action is mediated indirectly through secretion of progestogens by another endocrine organ. Results here described demonstrate that progestational response to amphenone in the rabbit is dependent on the adrenal glands.

Materials and methods. A modified Clauberg rabbit test was used for determination of progestational activity. Female New Zealand white rabbits from a single colony were used. Initial body weight range was 600-800 g. Ten mcg of estradiol-17 beta were administered in 0.25 ml sesame oil solution daily for 6 days followed by 5 days treatment with the test compound. Amphenone was given in 1 ml aqueous solution for each 100 mg administered. Progesterone, corticosterone and 11 β -hydroxyprogesterone were given in 0.2 ml sesame oil solution daily. All injections were subcutaneous unless otherwise specified. Animals were killed with chloroform 20 to 24 hours after the last injection. Body, uterus and adrenal (intact animals) weights were recorded. Uteri and adrenals were fixed in Bouin's solution for histology. Sections were stained with hematoxylin and eosin. The standard scale of McPhail(4) was used in rating progestational proliferation of the endometrium. Quantitative variability in this

assay has led to use of 0.5 mg progesterone as a standard for full endometrial proliferation (5). A 2-stage bilateral adrenalectomy and ovariectomy were performed with an interval of 8 to 10 days between each procedure. A lateral approach was used. Intravenous nembutal anesthesia (30 mg/kg) was supplemented with ether as required. Pre-operative treatment consisted of 25 mg hydrocortisone sodium succinate given intravenously 30 minutes to 1 hour before the second adrenal was removed. A 1% saline drinking solution was given *ad libitum* as post-operative treatment. Testing of compounds for progestational activity was not begun for at least one week following the second operation. Completeness of ovariectomy and adrenalectomy was determined at autopsy.

Results. Comparison of the progestational activity of amphenone with that of progesterone was made using unoperated rabbits. These results are shown in Series I (Table I). In this experiment, a total dose of 3.0 g amphenone produced effects comparable to 0.5 mg progesterone. A total dose of 2.0 g amphenone did not evoke the maximum progestational effect.

In several experimental series, the progestational effect of amphenone was compared

with that of progesterone in the adrenalectomized, ovariectomized rabbit. Series II contains results from a representative experiment. Amphenone failed to elicit a progestational response in the endometrium of the adrenalectomized, ovariectomized animal although progesterone produced its characteristic effect under these conditions. Ovariectomy alone did not prevent the progestational response to amphenone.

To determine completeness of adrenalectomy, animals were examined for accessory adrenals at autopsy. In 20 animals only one was found to have an accessory adrenal gland.

To further test the completeness of adrenalectomy, saline was withdrawn from an adrenalectomized, ovariectomized group after amphenone treatment was concluded. The 4 animals died 6 to 12 days after removal of this supportive measure.

In additional experiments (Series III), intact rabbits received a saline suspension of 2.5 mg cortisone acetate daily beginning on the first day of estrogen priming and continuing throughout treatment with amphenone or progesterone. Adrenals from rabbits receiving this dose of cortisone alone showed a 30 to 35% weight loss after 11 days. Cortisone completely suppressed the progestational response to amphenone. At the same dose level, cortisone did not prevent a progestational response to progesterone or a combination of amphenone and progesterone.

An increase in endogenous ACTH production was considered a probable factor in the progestational response to amphenone in the rabbit. Data on the effects of ACTH treatment of the intact rabbit are presented in Series IV. A total dose of 500 mg ACTH did not produce progestational changes in the rabbit endometrium. The adrenal weight range was 182-299 mg in the ACTH-treated group as compared with 66-91 mg in the progesterone-treated group. Total doses of 50 mg ACTH were likewise without effect.

At the total dosage indicated, the following steroids failed to yield a positive response in the Claiberg test using 3 animals for each compound: corticosterone (25. mg), 11 β -hydroxyprogesterone (10. mg), 17 hydroxycorticosterone acetate (25. mg), cortisone acetate

(50. mg). Ten mg of deoxycorticosterone acetate produced a maximum response comparable to that of 0.5 mg progesterone.

Discussion. Progestational proliferation of the rabbit endometrium in response to amphenone treatment has been demonstrated(1). Following ovariectomy and adrenalectomy, for removal of endogenous progestogen sources, the progestational response to amphenone did not occur although exogenous progesterone exerted its typical uterine effect. It has long been known that progesterone exerts the characteristic uterine effects in the absence of the ovaries(6). Similarly, ovariectomy had no effect on uterine response to amphenone. The progestational response to amphenone, therefore, is dependent on the secretion of the adrenal glands. Biological evidence for an adrenal cortical source of progestogens was obtained in 1930 by Englehart(7). Beall and Reichstein later reported the isolation of crystalline progesterone from adrenal cortical extracts(8).

Lyons *et al.*(9) have reported that treatment with highly purified ACTH preparations sensitizes the uterus of hypophysectomized, ovariectomized rats to the formation of deciduomata. These results indicate that ACTH-stimulated adrenals secrete progestogens.

The suppression of the progestational effects of amphenone by means of cortisone suggested that a decreased rate of secretion of ACTH was responsible for the ineffectiveness of amphenone. Cortisone is known to suppress ACTH production and thereby inhibit the biogenesis of adrenal steroids, including the progestogens. An elevated ACTH level in response to amphenone treatment was considered as a possible explanation for excess adrenocortical progestogen secretion. Decreased synthesis of corticosterone following amphenone has been reported(10). The increase in ACTH secretion subsequent to a reduction in corticosterone level could lead to excess progestogen production. However, in the present experiments, levels of ACTH which caused 2 to 3 fold increments in adrenal weight were incapable of inducing progestational changes in the rabbit endometrium.

Amphenone inhibition of specific enzyme systems in the adrenocortical biosynthetic

pathway of the rabbit would explain the progestational action. Amphenone has been reported to inhibit the synthesis of corticosterone, hydrocortisone, and cortisone in calf adrenal perfusion experiments(10). A block in the biosynthetic pathway between progesterone and corticosterone could result in excess secretion of a potent progestogen in amounts sufficient to induce progestational proliferation of the rabbit endometrium. Adrenocortical progesterone is probably the active progestogen since comparison with the principal adrenal corticosteroids in the Clauberg assay indicated that progesterone was much more potent than any of the active compounds.

Summary. Progestational proliferation of the rabbit endometrium in response to amphenone has been shown to be dependent on the adrenals. The progestational effect of amphenone was prevented by adrenalectomy as well as by cortisone treatment. Large doses of ACTH failed to produce progestational changes in the rabbit endometrium. It was suggested that amphenone exerts its progesta-

tional effect on the rabbit uterus by inhibition of specific enzyme systems in the adrenocortical biosynthetic pathway resulting in excess progestogen secretion.

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Genetic Control of Amylase Levels of Mouse Submaxillary Glands.* (23974)

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The amylase level of the submaxillary gland of certain rodents has been shown to be, at least in part, under hormonal control (1). Recent work has suggested that the autonomic nervous system may also play a role in regulating formation of amylase in rodents(2). The extent to which genetic control is involved in regulation of amylase levels is only generally suggested by data which show significant species differences in amylase levels of the submaxillary glands of rabbits, guinea pigs, rats and mice (unpublished data); such differences, however, may be an indirect reflection of genetic control of structural variation in glands of the several species.

It was, therefore, considered of importance to determine the role that hereditary mechanisms may have in controlling quantitative differences in amylase level. Furthermore, although proof of a genetic control of biochemical reactions has been demonstrated in certain microorganisms(3,4), little data of such a nature are available concerning such control in higher animals(5). Accordingly, to delineate the genetic role in amylase production, inbred lines of mice, where genetic uniformity is maximum, and where quantitative differences are therefore readily demonstrable (6), were used.

Methods. Adult male mice, 2-4 months old, were used. The animals were fasted for 48 hours, and, under Nembutal anesthesia, the submaxillary-sublingual glands were re-

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TABLE I. Amylase Levels of Mouse Submaxillary-Sublingual Glands.

Strain	No. of animals	Amylase level
CFW	22	.15 $\pm$.012*
C57BL/6	14	.14 $\pm$.006
C3H	35	.19 $\pm$.008
DBA/1	41	.10 $\pm$.003
F ₁ (C3H-DBA/1)	15	.15 $\pm$.008

* Mean $\pm$ stand. error of mean.

moved, weighed rapidly on a torsion balance, and frozen for future amylase analysis. For analysis, the glands were homogenized and the amylase content of appropriately diluted homogenate was then determined by the method of Myers, Free and Rosinski(7). Amylase activity is expressed in terms of milligrams of reducing sugar formed/mg wet weight of gland. Inbred mice of strains C57BL/6, C3H and DBA/1 (Jackson Memorial Laboratory) and CFW were used. C3H females were mated with DBA/1 males, and the submaxillary-sublingual glands from the resulting hybrid animals were analyzed for amylase content. Representative gland samples from each strain and from the C3H-DBA/1 hybrid were fixed in Bouin's solution, sectioned and stained, either with haematoxylin and eosin, or with Mallory's stain and examined for relative distribution of connective tissue elements.

Results. From Table I, it is apparent that statistically significant differences in amylase level of submaxillary-sublingual glands exist among the several inbred strains indicated. It is also clear that mating of the strain with highest amylase level (C3H, .19 mg reducing sugar/mg wet tissue) with that strain having the lowest amylase level (DBA/1, .10 mg) produced an F₁ hybrid generation with submaxillary gland amylase content intermediate in value (.15 mg). The difference in amylase level between the F₁ and the parent C3H and DBA/1 animals is statistically significant, with P values in all cases less than .0001. A significant difference also exists between mean values in the parent groups ($P < .0001$).

The C3H-DBA/1 hybrids, all of which resemble the parent C3H in hair color and body size, were mated among themselves and a wide range of hair color and amylase level was found in these F₂ animals. The mean amy-

lase levels of the 36 F₂ males obtained may be grouped as follows: 14/36, $M \pm SE = .15 \pm .008$; 9/36, $M \pm SE = .18 \pm .009$; 5/36, $M \pm SE = .33 \pm .012$; and 8/36, $M \pm SE = .07 \pm .009$.

The characteristic strain difference in amylase level is probably not the result of genetic differences in amount of connective tissue or number of ducts and acini in glands of the several strains. Histological examination of glands from each mouse strain showed no significant differences in relative distribution of these elements. It may be concluded, therefore, that the amylase level characteristic of each strain, under the experimental conditions selected, is genetically determined. The precise mechanism of this control, however, can only be suggested, since the number of animals employed was limited. The data, however, are consistent with a gene control that is multiple and additive, possibly with each dominant allele producing an amylase quantity in the approximate range .05-.07 mg reducing sugar/mg wet gland. On this basis, the parental genotypes for amylase may be represented as AABbCc for C3H (amylase level = .19 or approximately 4 x .05), aabbCC for DBA/1 (amylase level = .10 or approximately 2 x .05), and AaBbCc for the hybrid (amylase level = .15 or approximately 3 x .05).

Summary. Under uniform conditions of age, sex and environment, the concentration of the specific enzyme, amylase, present in mouse submaxillary-sublingual glands varies with the strain, the amounts being (amylase activity expressed as mg of reducing sugar/mg wet tissue) CFW, .15 mg; C3H, .19 mg; C57BL/6, .14 mg; and DBA/1, .10 mg. The characteristic amylase level is probably not the result of genetic differences in amount of connective tissue or number of ducts and acini in glands of the several strains as histological examination of representative tissues of each mouse strain shows no significant differences. Thus, the submaxillary gland amylase level for each strain is genetically determined.

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Influence of Steroids Upon Osmotic Pressure Changes in Isolated Frog Skin Pouches.*† (23975)

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It was reported(1) that various steroids, added to the interior of pouches prepared from skin of frog legs, suspended in and filled with frog Ringer's solution, were capable of raising the osmotic pressure on the inside of the pouch in experiments lasting up to 16 hours. Similar pouch preparations from the opposite leg of the same animal to which no steroid or just the solvent was added served as controls. The ability of surviving frog skin to transfer sodium across the membrane had been demonstrated before(2,3,4). It was assumed, from our experiments, that the further increase of osmotic pressure after addition of appropriate steroids was an expression of a direct action of such steroids upon electrolyte transfer at the cellular level. No measurements of electrolytes were performed in our previous investigations. Steroids which exhibited the most pronounced effect were: desoxycorticosterone, 9 α -fluorohydrocortisone, hydrocortisone and, to a much lesser degree, estriol. Testosterone showed no effect. No significant changes in fluid volume inside the skin pouch were observed, despite the change of osmotic pressure.

Methods. In experiments to be reported here, the action of other steroids under similar

experimental conditions was tested. All experiments were again performed on skin pouches prepared from hind legs of frogs (*Rana pipiens*), the epithelial layer remaining on the outside. Steroids in various concentrations were added to preparations from one leg, the other leg serving as control. Methods of dissolving steroids, determining freezing point depression in a Fiske osmometer etc., remained the same, as previously published(1). Most experiments were terminated after 8 hours. Sodium and potassium measurements were performed with the flame photometer. Data were computed in relation to total surface of skin pouch, rather than per cm². This is permissible, since in a sequence of 50 unselected preparations, the mean surface of the actively exposed skin was 16.37 cm² with standard deviation of only 0.58 and standard error of mean of 0.081. The steroids tested were: dl-alldosterone, 2-methyl-9 α -fluorohydrocortone, prednisone and prednisolone. The first 2 were chosen because of their known strong effects upon electrolyte shifts, the latter because of their relative inertness.

Results. Table I illustrates the effects of these substances upon osmotic pressure. Dl-alldosterone and 2-methyl-9 α -fluorohydrocortone both exhibited a striking effect resulting in a considerable increase in osmolarity of the fluid in the treated pouch as compared with control preparation. In general, the higher the steroid concentration, the more potent was the effect, with the exception of the ex-

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TABLE I. Osmotic Pressures (in Milliosmols/l) of Fluids inside Skin Pouches in 8 Hr Experiments, Expressed as Increases above Initial Concentration of about 200 Milliosmols/l.

Steroid	$\mu\text{g/cc}$	No. of animals	Control*			Treated*			Δ , mean
			Mean	S.D.	S.E.	Mean	S.D.	S.E.	
DL-aldosterone	.2	23	15.5	4.6	.9	94.2	49.3	10.3	+ 78.7
	1.0	15	22.5	5.6	1.5	126.5	74.6	19.2	+104.0
2-Methylfluorohydrocortone	.02	7	18.5	2.5	.9	37.1	11.1	4.2	+ 18.6
	.2	7	18.9	2.0	.8	181.3	17.6	6.7	+162.4
	.25	11	19.8	5.0	1.5	206.6	42.5	12.8	+186.8
	1.0	5	14.6	4.2	1.9	131.6	80.7	36.1	+117.0
Prednisone	2.0	10	14.2	3.6	1.1	15.8	5.6	1.5	N.S.
	20.0	10	24.7	4.5	1.4	21.2	6.0	1.9	"
Prednisolone	2.0	10	26.7	3.6	1.1	25.9	2.9	.9	"
	20.0	10	21.0	3.1	1.0	33.8	1.7	.5	+ 12.8

* Mean, stand. dev., and stand. error of mean are given.

N.S. = Not significant.

periment in which the concentration of 2-methylfluorohydrocortone was highest (1 $\mu\text{g/cc}$). However, in this experiment, the number of observations was small and the standard deviation as well as standard error of the mean relatively high. Prednisone had no effect upon osmolality in concentrations much higher than other steroids. Prednisolone exhibited only a slight increase when administered in a concentration as high as 20 $\mu\text{g/cc}$. The fluid volumes, inside the skin pouch measured gravimetrically, increased considerably under the influence of dl-aldosterone and the 2-methyl-9 α -fluorohydrocortone (Table II). In some specimens the fluid content had nearly doubled at conclusion of the experiment. This could be readily observed by bulging of skin

pouches compared to the controls. No significant difference in concentration of sodium, and possibly a slight increase in potassium concentration in most experiments was observed inside the skin pouch (Table III). However in view of increased volume of fluid, the total content of these electrolytes has risen considerably under the influence of the hormone.

The increase in milliosmols/liter was evidently not explained by changes in sodium or potassium concentrations, which represented the major components of Ringer solution. One must assume, therefore, that elevation of osmotic pressure under the influence of the steroids must have resulted from a discharge of osmotically active molecules from the frog skin itself, since not enough excess of electro-

TABLE II. Volumes (cc) of Fluids inside Skin Pouches in 8 Hr Experiments, Expressed as Increases above Initial Volume of 4 cc.

Steroid	$\mu\text{g/cc}$	No. of animals	Control*			Treated*			Δ , mean
			Mean	S.D.	S.E.	Mean	S.D.	S.E.	
DL-aldosterone	1.0	15	.33	.28	.07	2.03	.54	.14	+1.70
2-Methylfluorohydrocortone	.02	7	.41	.11	.04	.64	.10	.04	+ .23
	.2	7	.48	.10	.04	3.40	.31	.12	+2.92
	.25	11	.45	.21	.06	3.98	.61	.18	+3.53

* Mean, stand. dev., and stand. error of mean are given.

TABLE III. Sodium and Potassium (ME/l) in Fluids inside Skin Pouches in 8 Hr Experiments. Each figure represents values obtained in pooled specimens from 5 skin pouches.

Steroids	Cone., $\mu\text{g/cc}$	Control		Treated	
		Na	K	Na	K
2-Methylfluorohydrocortone	1	114.1	1.08	108.1	1.08
	1	99.2	1.13	101.6	1.28
	1	102.4	1.16	100.8	1.25
	1	100.0	1.15	99.8	1.32
DL-aldosterone	1	101.6	1.32	100.8	1.77

lytes was present in the solution inside the skin bag to account for this phenomenon. No significant differences were found in concentration of total protein, amino acids, urea, hexosamines or uronic acid. Further investigations are being performed to characterize the contents of the fluids. That the increase in osmotic pressure is directly related to a specific action of certain corticoids is strongly suggested by the observation that other steroids tested had no such effect. The nature of the material released by the active steroids might offer some insight into the mechanism of steroid-promoted water and electrolyte transfer.

Summary. The influence of dl-aldosterone, 2-methyl-9 α -fluorohydrocortone, prednisone and prednisolone upon osmotic pressure of fluids inside of isolated frog skin pouches in

a Ringer bath was tested. A considerable increase of osmolarity and increase in total fluid volume as the result of the action of the first 2 steroids was observed. The sodium and potassium concentrations remained unchanged. The significance of this observation was briefly discussed in terms of appearance of osmotically active material from the skin under the action of certain steroids.

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Effect of Shock on Rat Heart and Brain Mitochondria. (23976)

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From earlier work, it is known that changes in metabolic pattern and concentration of metabolites of brain and heart occur under the influence of anoxia and fatigue(1). Similarly, changes induced by shock in various organs and tissues may be caused by anoxia and electrolyte imbalance which is part of the shock syndrome(2). Since this suggests a disruption of the enzymatic pattern in the cell, it was of interest to study the effect of shock on mitochondria. The efficiency of phosphorylation processes was investigated because failure of energy metabolism has been implicated in irreversibility of shock.

Methods. Irreversible shock was produced in 200-250 g male rats of Sprague-Dawley strain by the tourniquet, Noble-Collip drum and hemorrhagic technics. A degree of shock which resulted in a mortality of 80-90% in 24 hours, was employed. The animals were sac-

rificed 1-2 hours after induction of shock and mitochondria were prepared from heart(3) and brain(4). Mitochondria were pooled from 6 control and 6 experimental animals, except for hemorrhagic shock when only one animal was available for each experiment. Polarographic assay for oxidative phosphorylation in heart mitochondria was carried out as previously described(5). Adenosine-triphosphatase activity was assayed according to Zetterstrom and Ernster(6). The incorporation of P³² labelled inorganic phosphate into adenosine-triphosphate was done in Warburg apparatus at 18°C. The reaction mixture (3 ml) contained phosphate buffer (10 mM at pH 7.6), MgCl₂ (1mM), adenosine diphosphate (3.3mM), sucrose (0.30M), P³² inorganic phosphate (750,000 counts/minute), succinate (20mM) where indicated, and heart mitochondria. For brain experiments, malonate (10mM) and NaF (10mM) were also added, sucrose was 0.25M, and glutamate (20mM) where indicated. The experiment

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TABLE I. Effect of Drum Shock on Incorporation of P^{32} Labelled Inorganic Phosphate into Adenosine-Triphosphate.

	O_2 uptake, μ moles	ATP, μ moles	ATP, counts per min.	ATP, counts per μ mole
<i>Heart</i>				
Normal:				
No addition	.0	1.173	2,400	2,048
Succinate	4.07	1.372	32,100	23,420
Drum shock:				
No addition	.0	1.289	600	466
Succinate	3.50	1.415	11,700	8,270
<i>Brain</i>				
Normal:				
No addition	.86	2.122	4,425	2,082
Glutamate	1.02	2.070	6,680	3,230
Drum shock:				
No addition	.70	2.610	5,100	1,954
Glutamate	1.02	3.095	3,000	969

was stopped after 15 minutes by addition of perchloric acid and ATP was isolated by the ion-exchange method of Cohn and Carter(7) and its radioactivity counted. For electrolyte analysis, heart mitochondria pooled from 6 normal and 6 shocked animals were prepared simultaneously in 0.32M sucrose. Kjeldahl nitrogen analyses were performed on mitochondrial suspensions and the results expressed as protein by multiplying by 6.25. Following this, the suspensions were digested with nitric acid, ashed, and the electrolyte analyses performed. Sodium and potassium were determined by flame photometry, phosphorus by the Ennor and Stocken method(8), magnesium by a modification of the Heagy method(9), and calcium by a modification of the Ashizawa method(10).

Results. Table I shows that a clear difference exists in incorporation of inorganic phosphate in ATP in heart and brain mitochondria from normal and drum shocked rats. In shock, the incorporation was decreased 73% in heart and completely suppressed in brain. In parallel studies, these findings were confirmed by showing that specific activity changes in inorganic phosphate were inversely related to the ATP changes. The rate of respiration in normal and shocked preparations do not appreciably differ. In other experiments, the concentration of respiratory en-

zymes of normal and shocked preparations is identical, which agrees with the similar rate of respiration observed.

Since some diminution in ATP turnover and hence phosphate incorporation could have been due to decreased adenosine-triphosphatase activity, this activity was subsequently examined in normal and drum shocked preparations (Table II). No significant difference in activity was found. Activity in both heart and brain preparations was promoted by magnesium and calcium and was relatively stable to storage. Thus differences in P^{32} incorporation suggested an impairment of ATP synthesis.

These findings were extended by employing heart mitochondria of normal animals and those in which 3 different types of shock were

TABLE II. Adenosine-triphosphatase Activity of Heart and Brain Mitochondria.

	μ moles phosphate formed/hr/mg protein			
	Heart		Brain	
	Normal	Shock	Normal	Shock
No addition	.45	.35	7.37	6.62
MgCl ₂ (5 mM)	3.34	3.47	27.41	14.71
CaCl ₂ "	2.91	3.31	25.10	
MgCl ₂ + CaCl ₂	3.91	4.97		
+ MgCl ₂ (5 mM)				
Fresh preparation	9.42	9.00	7.80	7.14
Aged 4 days at 2°C	7.20	7.14	3.42	5.22

induced. Table III summarized data from the phosphorus to oxygen ratios obtained by the polarographic method(5) during respiration of alpha-ketoglutarate. The data show that the efficiency as well as net synthesis of ATP are lowered in heart preparations from the 3 types of shock employed.

Since profound electrolyte changes accompany the onset of shock *in vivo*(2), it seemed possible that a changed electrolyte distribu-

TABLE III. Effect of Different Types of Shock on Oxidative Phosphorylation in Heart Mitochondria.

Type of rat	P/O	μ moles ATP synthesized/sec./mg protein/l	No. of exps.
Normal	3.59	4.4	25
Tourniquet shock	3.08	3.4	9
Drum "	3.07	3.9	5
Hemorrhagic "	2.45	2.7	14

TABLE IV. Electrolyte Analysis of Rat Heart Muscle Mitochondria.

	μ moles/mg protein		Significance* "P"
	Normal	Shock	
Calcium	.114	.090	.10
Phosphorus	.552	.650	.05
Magnesium	.039	.045	.10
Sodium	.101	.069	.10
Potassium	.128	.154	.10

* Calculated by method of paired comparisons. Each value represents avg obtained from 30 hearts.

tion might be evident in mitochondria of shocked rats. This had further interest because of the well-known role which electrolytes have in phosphorylation reactions. The electrolyte content of normal and drum shocked heart preparations is reported in Table IV. The greatest difference was a 15% increase in total phosphorus content in shock. Also, a change in concentration of sodium and potassium was found in shock mitochondria, sodium having decreased and potassium increased. The magnesium content was little changed and some decrease in calcium was found in shock preparations.

Discussion. It appears certain that phosphorylation processes in mitochondria isolated from animals in shock are impaired. The data reported here are in close agreement with those of Strawitz and Hift(11,12) who observed lower P/O ratios in dog heart mitochondria obtained from animals in hemorrhagic shock. In agreement with these investigators we also found no differences between mitochondria of normal and shocked animals with respect to respiration rate and content of respiratory enzymes. Strawitz and Hift obtained photomicrographs which indicated that heart mitochondria from shocked animals differed in size and shape from normal mitochondria. It is well known that swelling and shrinking of mitochondria causes changes in metabolic activity(13,14). Since structural and functional changes in mitochondria are influenced by the electrolyte environment(13), we undertook a study of electrolyte distribution in normal and shocked preparations.

Although Slater and Cleland(15) have shown that calcium ions cause a decline in phosphorylative activity in rat heart mito-

chondria, this mechanism would not account for the lower P/O ratios of shocked preparations since increased concentration of these ions was not found. However, Hunter and Ford(14) reported a decreased phosphorylation in the presence of higher phosphate concentrations, and it may be that the increased phosphate content in shocked preparations is the cause of the lower P/O values. The elevated mitochondrial phosphate content may be related to increases in serum phosphate observed in shock(2). The altered distribution of sodium and potassium in these preparations also may be responsible for changes in phosphorylation processes. The changed pattern of electrolyte distribution is consistent with the structural changes reported by Strawitz and Hift(11,12). Thus, the electrolyte changes appear responsible for the structural and functional changes in mitochondria of animals in shock.

Summary. Phosphorylation processes were studied in heart and brain mitochondria of rats subjected to severe shock by the Noble-Collip drum, tourniquet and hemorrhage techniques. A decreased synthesis of adenosine-triphosphate was found in drum shock. Lowered P/O ratios were obtained with heart mitochondria of all 3 types of shock when compared to normal preparations, and the adenosine-triphosphatase activity was unchanged. After shock, the electrolyte composition of heart mitochondria was altered; the total phosphorus and potassium increased while sodium decreased and magnesium and calcium showed only slight differences. The relationship of these findings to the structure and function of mitochondria in shock are discussed.

We wish to thank Dr. Peter Van Soest for the cation determinations.

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Antiparasitic Drugs. II. Anticoccidial Activity of 4,5 imidazoledicarboxamide and Related Compounds. (23977)

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Coccidiosis is an important protozoan disease of poultry which "costs poultry raisers millions of dollars each year because of unthriftness, poor utilization of feed, loss of weight, and death of infected birds" (1). During the past 2 decades several effective chemotherapeutic agents have been discovered for prevention and control of coccidiosis in poultry and other domestic animals. Nevertheless, there has been a constant search for more effective and better tolerated drugs for use as coccidiostats. We recently found that 4,5-imidazoledicarboxamide (2) (Fig. 1) is a potent and well-tolerated coccidiostat. The compound has been assigned the generic name of glycarbylamide.* This paper reports the results of chemical and biological studies on glycarbylamide and related compounds.

Materials and methods. *Chemical.* 4,5-Bis (*N*-acetylcarbamy)-imidazole (V). Two g of 4,5-imidazoledicarboxamide was refluxed for 15 hours in a mixture of 100 ml of acetic anhydride and 10 ml of acetic acid. The reaction mixture was then concentrated to dryness *in vacuo* at 100°. The residue thus obtained was slurried with water, the slurry allowed to stand at room temperature for about an hour and then filtered. Two g of product

was obtained. After recrystallization from methanol it melted at 259-60°. *Anal.* Calcd. for $C_9H_{10}O_4N_4$: C, 45.38; H, 4.23; N, 23.53; N.E. 238. Found: C, 45.42; H, 4.26; N, 23.77; N.E. 241. 4,5-Bis (*N*-propionylcarbamy)-imidazole (VI). This compound, prepared by the same general method as V, melted at 239-40° after recrystallization from ethanol. *Anal.* Calcd. for $C_{11}H_{14}O_4N_4$: C, 49.61; H, 5.30. Found: C, 49.36; H, 5.27. 4-Acetylcarbamy-5-imidazolecarboxamide (IV). Two and four-tenths g of 4,5-bis (*N*-acetylcarbamy)-imidazole was suspended in 25 ml of water and 10 ml of *N* sodium hydroxide was added with vigorous shaking. The clear solution obtained stood at room temperature for about 60 hours during which time the product slowly precipitated. When

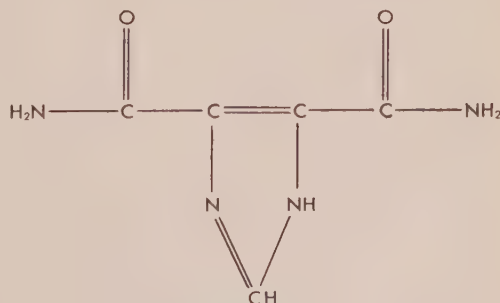


FIG. 1. 4,5-Imidazoledicarboxamide (Glycarbylamide).

* 'GLYCAMIDE' is the trademark of Merck & Co., for this compound.

filtered and recrystallized from methanol, it melted at 285°. *Anal.* Calcd. for $C_7H_8O_3N_4$: N, 28.56. Found: N, 29.19. *N*⁴,*N*⁵-Dimethyl-4,5-imidazoledicarboxamide (IX). This was prepared from 4,5-imidazoledicarboxylic acid dimethyl ester and 40% aqueous methylamine; m.p. 223°. *Anal.* Calcd. for $C_7H_{10}O_2N_4$: C, 46.14; H, 5.53. Found: C, 46.38; H, 5.33. 4,5-Imidazoledicarbonitrile (XII). A mixture of 10 g of 4,5-imidazoledicarboxamide and 60 ml of phosphorus oxychloride was heated for 2 hours on a steam bath. Excess phosphorus oxychloride was then removed *in vacuo*. Sufficient ice water was added to the residue to permit mixing. Sodium bicarbonate was then added until only a weak acid reaction was observed. The cold mixture was filtered. The precipitate gave 7 g of product on extraction with hot ether. Extraction of filtrate with ether yielded an additional 1.5 g. The combined extractives, recrystallized from 25 ml of boiling water, gave 5.3 g of product, softening at 173°, melting at 176-177°. Woodward(3) reports a melting point of 175°. 1-Ethyl-4,5-imidazoledicarboxamide (XIV). To a solution prepared by dissolving 3.6 g of sodium metal in 900 ml of methanol were added successively 27.3 g of methyl-4,5-imidazoledicarboxylate and 25 g of ethyl iodide. The mixture was refluxed for 16 hours, then concentrated *in vacuo* to a syrupy liquid. The liquid was treated with 300 ml of concentrated ammonium hydroxide, mixed and let stand 60 hours. After this time the precipitated solid was filtered off, washed with a little cold water and air dried. The yield of crude product was 17 g, m.p. 215°. The material was dissolved in 850 ml of boiling absolute alcohol, charcoaled and filtered. After concentration to about two-thirds volume, the solution when cooled in an ice bath precipitated 12.1 g of 1-ethyl-4,5-imidazoledicarboxamide, m.p. 215-6°. *Anal.* Calcd. for $C_7H_{10}O_2N_4$: N, 30.75. Found: N, 30.89. 1-Benzyl-4,5-imidazoledicarboxamide (XV). To a solution prepared by dissolving 6 g of potassium metal in 250 ml of methanol were added successively 25 g of methyl-4,5-imidazoledicarboxylate and 26 g of benzyl bromide. The mixture was refluxed with stirring for 9

hours. Precipitated potassium bromide was recovered by filtration. The filtrate was concentrated *in vacuo* to a syrupy liquid and to this was added 300 ml of concentrated ammonium hydroxide. After standing 48 hours, precipitated solid was filtered off. The air-dried product was dissolved in 1.8 L of hot acetone, a small amount of insoluble material being rejected. The acetone solution was concentrated and filtered. Crystallization gave 21.6 g of product melting at 203-5°. On recrystallization from methanol the 1-benzyl-4,5-imidazoledicarboxamide melted at 204-6°. *Anal.* Calcd. for $C_{12}H_{12}O_2N_4$: N, 22.94. Found: N, 23.14.

Biological. Experimental cecal coccidiosis was produced in 2-week-old chicks as follows: Straight run White Leghorn chicks, in groups of 3 each, were weighed and placed in cages with wire floors. They were fed *ad lib.*, a standard laboratory ration in which graded concentrations of test-chemicals were carefully blended just prior to use. Nicarbazine (4) was used as the reference standard. The normal and infected control birds were fed basal ration. On the second day of test, the chicks were each orally inoculated with 50,000 sporulated oocysts of *Eimeria tenella*. Papers under cages were examined on 6th, 7th and 8th days of assay for presence or absence of bloody droppings. A score of 0 was given if no bloody spots were observed; scores of 1, 2 or 4 were assigned for 1-3, 4-6 and >6 bloody spots, respectively. On 8th day of assay, the surviving birds were weighed, sacrificed and examined grossly for cecal coccidiosis lesions. To eliminate bias, the identity of groups under examination was not known to the observer. Normal ceca were scored 0 and ceca with detectable, moderate or maximal lesions were scored, 1, 2 and 4, respectively. When a bird died, and cecal coccidiosis lesions were present, a score of 5 was recorded. If the total of the 2 scores was 0-5, a rating of "active" was given to the compound; if the total score was 6 or more the compound was rated "inactive," at the concentration tested.

Results. The relative anticoccidial potency of 17 imidazole compounds is shown in Table I. None was as potent as 4,5-imidazoledicar-

TABLE I. Anticoccidial Potency of 4,5-Imidazoledicarboxamide and Related Compounds.

Com- pound	Substituents on imidazole ring				% in feed	Score rating, active & inactive	% relative potency (compared to 4,5-imida- zoledicar- boxamide)	Refer- ences
	1	2	4	5				
								
I	H	H	H	H	.2	I†	< 1	†
II	H	H	CONH ₂	H	.2	A	1	5
III	H	H	"	CONH ₂	.002	A	100	2
IV	H	H	CONHCOCH ₃	"	.008	A	25	*
V	H	H	"	CONHCOCH ₃	.004	A	50	*
VI	H	H	CONHCOC ₂ H ₅	CONHCOC ₂ H ₅	.004	A	50	*
VII	H	H	COOH	COOH	.2	I	< 1	†
VIII	H	H	COOCH ₃	COOCH ₃	.2	I	< 1	2
IX	H	H	CONHCH ₃	CONHCH ₃	.05	I	< 4	*
X	H	H	CONHNH ₂	CONHNH ₂	.02	I	< 10	6
XI	H	H	CONHNHCO	"	.02	I	< 10	6
XII	H	H	CN	CN	.04	I	< 5	3, *
XIII	CH ₃	H	CONH ₂	CONH ₂	.02	A	10	2
XIV	C ₂ H ₅	H	"	"	.005	A	40	*
XV	C ₆ H ₅ CH ₂	H	"	"	.02	A	10	*
XVI	β-D-ribo- furanosyl	H	"	"	.01	I	< 20	7
XVII	H	CH ₃	"	"	.04	I	< 5	8

* Described in this paper.
I = inactive.

† Commercial sample, Eastman Kodak Co.

‡ A = active,

boxamide (III), which was active at 0.002% concentration in the feed. Imidazole itself had no activity; imidazole-4-carboxamide (II) was much less potent than the dicarboxamide. In compounds IV, V and VI, derivatives of 4, 5-imidazoledicarboxamide in which one or both amide groups were acylated, activity was retained, but other 4,5-substituted imidazoles closely related to the dicarboxamide, 4,5-imidazoledicarboxylic acid and its dimethyl ester (VII, VIII), the bis (N-methyl-carboxamide) (IX), the dihydrazide (X) and cyclic hydrazide (4,7-dihydroxy-1,3,5,6-tetraazaindene, XI) and the dinitrile (XII), were all completely inactive. Replacement of hydrogen at position 2 of 4,5-imidazoledicarboxamide by methyl, as in XVII, likewise resulted in loss of anticoccidial effectiveness. The 1-methyl and 1-ethyl derivatives of 4,5-imidazoledicarboxamide (XIII, XIV) were effective coccidiostats but less potent than glycarbylamide (III). Replacement of 1-methyl with 1-benzyl gave a diamide (XV) of equal effectiveness.

Glycarbylamide was approximately 4 times more potent than nicarbazin for *E. tenella*

and was highly effective for coccidial infections produced by *Eimeria acervulina* and *Eimeria necatrix* (not yet published.)

The possibility of interference with purine biosynthesis was our first consideration in connection with the mechanism of action of 4,5-imidazoledicarboxamide. Aside from the labile ribose phosphate unit, the dicarboxamide differs from 5-formamido-4-imidazole-carboxamide ribotide(9,10) only in the substitution of 5-CONH₂ for 5-NHCHO. However simultaneous administration of either 5-amino or 5-formamido-4-imidazolecarboxamide with 4,5-imidazoledicarboxamide had no effect upon the anticoccidial action of the latter compound. 1-β-D-Ribofuranosyl-4,5-imidazoledicarboxamide (XVI) was inactive at 0.01%. The possibility that the anticoccidial action of 4,5-imidazoledicarboxamide was due to a purine antagonist function was further tested by simultaneous administration of xanthine, hypoxanthine, guanine, adenine and uric acid and the coccidiostat. No effect on therapeutic potency was observed with any of these combinations.

Summary. 1. Preparation of 7 substituted

imidazoles has been described. 2. Anticoccidial activity was demonstrated for imidazole-4-carboxamide and certain related compounds. 3. The most potent compound was 4,5-imidazoledicarboxamide (glycarbylamide). 4. Replacement of one or both carboxamides in the 4,5 positions on the imidazole ring reduced or completely eliminated the anticoccidial activity of glycarbylamide. Substitutions at the 1 or 2 positions on the imidazole ring produced compounds of less activity than glycarbylamide.

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Further Properties of Isoglutamine-oxytocin; Inhibition of Pressor Activity of Vasopressin.* (23978)

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The synthesis of the isoglutamine isomer of oxytocin, the chief oxytocic principle of the posterior pituitary gland, has recently been described(1). This isomer, the cyclic disulfide of L-cysteinyl-L-tyrosyl-L-isoleucyl-L-isoglutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-leucyl-glycinamide, differs from oxytocin by substitution of an isoglutamine residue for the glutamine residue in the hormone. In view of this difference in structure and our interest in the correlation of the structures and properties of such polypeptides, the isomeric compound was compared with oxytocin. Observations were made on distribution coefficients, optical rotation, electrophoretic mobility, molecular weight and infrared pattern(1). In addition, the isomer was tested for some physiological activities associated with oxytocin; namely, oxytocic activity, avian vaso-depressor activity, and pressor activity in the rat. As has been already reported, these ac-

tivities were not detected in the isomer. Further testing revealed that isoglutamine-oxytocin exerts a definite inhibitory effect on the pressor action of administered vasopressin in the anesthetized rat. Inhibition toward both purified arginine-vasopressin and the posterior pituitary standard powder was observed. It may be recalled that oxytocin can be converted into a crystalline flavianate derivative (2). Efforts were therefore made to determine whether the isoglutamine isomer could show similar behavior. It has now been found that isoglutamine-oxytocin also yields a crystalline flavianate, and that the latter differs in form from the corresponding oxytocin derivative. When the acetate of isoglutamine-oxytocin was rigorously dried, it underwent change, as indicated by decrease in solubility. Results of elementary analysis and molecular weight determination suggested that it had probably lost acetic acid.

Experimental. Inhibition of pressor action of vasopressin. Anesthetized rats weighing 290-480 g were injected intravenously with solutions of isoglutamine-oxytocin(1), vasopressin, and various mixtures of these. The

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[†] Part of this work was done during tenure of Fellowship as Established Investigator of Am. Heart Assn.

conditions described by Lindquist and Rowe (3) for bioassay of posterior pituitary pressor activity were used. Mepesulfate 'Roche,' however, replaced the heparin. The U.S.P. Posterior Pituitary lobe powder with a stated potency of 2 U/mg, or a solution of highly purified arginine-vasopressin assayed against the latter, served as the reference pressor substance or standard. When a solution containing 180 γ of isoglutamine-oxytocin was injected, no pressor activity was observed. It was noticed, however, that a subsequent injection of standard containing 30 milliunits of vasopressin produced almost no effect on blood pressure. The response was only 6% of that given by the same dose of standard prior to administration of the isoglutamine compound. To evaluate this effect more carefully the following procedure was used. Isoglutamine-oxytocin was injected at the same time as the standard, *i.e.*, as a mixed solution containing both polypeptide and standard. Administration of the mixture was preceded and followed by injections of the standard alone. Injections were performed only after the blood pressure of the animal had returned to the baseline after the preceding one. The effect of isoglutamine-oxytocin on response to the standard in the mixture and on response to the subsequent standard was evaluated by comparing these responses with results of the initial injections of standard. If inhibition was observed, several subsequent injections of standard were made in an attempt to estimate the duration of the effect. When the results of the latter injections indicated that the animal had regained its sensitivity to the standard, the amount of isoglutamine-oxytocin in the mixture was then varied, and the series of injections was repeated. Because the inhibitory effect persisted for some time, only a limited amount of data was obtained from each rat. The mixtures of isoglutamine-oxytocin and standard were made up by adding different volumes (0.01-0.19 ml) of a 0.00067 molar solution of isoglutamine-oxytocin acetate to a given volume of the standard solution of vasopressin having a potency of 0.2 U/ml. Usually 0.15 ml of the latter solution, or 30 milliunits of pressor activity, was used. An attempt was made to work at a fixed level

of vasopressin and vary only the amount of the isoglutamine compound. However, because of some difference in sensitivity to vasopressin among the animals, other levels of standard were also used to obtain responses of suitable magnitude. In each experiment, however, the level of standard administered in the mixture was always kept the same as that of the preceding and following standards.

The *results* of a number of typical injections are given in Table I. Inhibition[‡] is expressed in terms of percentage decrease in response to a dose of vasopressin in the presence of a dose of isoglutamine-oxytocin. The relative doses of isoglutamine compound and vasopressin in each experiment are given in terms of μ g of the former/milliunit of pressor activity of the latter, *i.e.*, as a weight-activity ratio.

It was noticed that when isoglutamine-oxytocin was injected along with the standard at a ratio of approximately 0.7-1.8 γ /milliunit of pressor activity, the hypertensive response to the standard generally fell approximately 30-50%. In terms of relative weights of the 2 substances, this would correspond to 280-720 parts of isoglutamine peptide to one of vasopressin, based on an activity of approximately 400 U/mg for the latter. Some inhibition to subsequent injections of vasopressin persisted for about one-half to one hour. At a ratio of 0.4 γ /milliunit of pressor activity, no inhibitory effect was observed. At ratios above approximately 4-6 γ /milliunit, inhibition was

[‡] Inhibition in the rat of the pressor activity of arginine-vasopressin by preparations of the latter that had been treated with acetic anhydride has been observed recently (Cash, W. D., Studer, R. O., and du Vigneaud, V., in preparation).

[§] While this manuscript was in preparation, a nonapeptide having the structure CyS-Tyr-Tyr-Ileu-

$\text{Glu(NH}_2\text{)-Asp(NH}_2\text{)-Cys-Pro-Leu-Gly(NH}_2\text{)}$ was reported to inhibit the action of oxytocin on the isolated rat uterus and in lowering blood pressure of the chicken. (Guttmann, St., Jaquenoud, P. A., and Boissonnas, R. A., *Naturwiss.*, 1957, v24, 632). It is of interest that both the latter nonapeptide and isoglutamine-oxytocin possess cyclic disulfide rings that are larger than that which occurs in oxytocin and vasopressin.

INHIBITION OF VASOPRESSOR ACTIVITY

TABLE I. Inhibition of Pressor Activity of Vasopressin.

Rat No.	Wt, g	Dose of isoglutamine-oxytocin (I), γ	Dose of vasopressin (V), mU	Ratio I/V, γ /mU	Inhibition, %	Duration, min
1760*	360	14	20	.7	37	60
		18.7	31	.6	18	
		27.4	30	.9	24	
		19	21	.9	55	75
1761*	380	24	40	.6	16	<10
		22	30	.7	42	
		27	30	.9	30	
		36	40	.9	36	
1769*	350	55	31	1.8	55	
		121	51	2.4	70	
1768*	300	54	30	1.8	48	
		108	30	3.6	88	
1727*	320	102	30	3.4	88	
		136	20	6.8	Complete	>90
1733†	340	7	16	.4	None	
1731†	290	21	30	.7	60	
		48	30	1.7	62	
		68	30	2.3	85	
1816*†	330	53	29	1.8	78	
		108	30	2.9	Complete	
1812*†	390	53	29	1.8	55	
		108	30	3.6	62	<30

* Purified arginine-vasopressin served as standard.
 powder served as standard.

† U.S.P. posterior pituitary lobe
 ‡ A Sanborn electromanometer was used.

usually complete; *i.e.*, there was no response to the administered vasopressin. Duration of the effect generally increased markedly with degree of inhibition initially observed. The results were similar when either purified arginine-vasopressin or the U.S.P. Posterior Pituitary lobe powder served as the pressor substance.

Although only a limited number of observations were made in each rat, there frequently appeared to be a fair degree of discrimination between doses of isoglutamine peptide in any one animal. Some responses, however, were irregular. This may be due in part to irregularity in response to vasopressin. In the rat preparation used, successive injections of a constant dose of vasopressin yield responses that may differ by 15-20%. Moreover, levels of vasopressin used were somewhat high and may not have been optimum for regular responses(4). Other factors which may have had some bearing on the results are possible variations in depth of anesthesia among the animals (see discussion) and variations in animal weight.

In preliminary experiments, isoglutamine-oxytocin also appeared to show some inhibition of the action of oxytocin on the rat uterus. This observation, however, needs further study and confirmation.

Formation of a crystalline flavianate. To a solution of 4 mg of polypeptide acetate in 0.19 ml of water was added 0.12 ml of a 5% solution of flavianic acid. Slow evaporation of the solution in the cold to a small volume yielded highly refractive rectangular and elongated plates. Under similar conditions oxytocin yields a flavianate in the form of fine, silky needles(2). The crystals were separated by centrifugal filtration in the cold, washed in a similar manner with 2 drops of 0.25% flavianic acid, and dried in an evacuated desiccator in the cold. After drying, the material became irregular in shape but remained highly refractive. It decomposed gradually above 205°. A sample was hydrolyzed in 6 N HCl for 14 hours at 120°. Paper chromatography of the hydrolyzed material in *n*-butyl alcohol-water-acetic acid (5:1:5) followed by 75% phenol, and development with ninhydrin, showed the

presence of the expected amino acids.

Change on Drying. Isoglutamine-oxytocin acetate had been obtained by lyophilization of the polypeptide from a dilute solution of acetic acid(1). The residual white, fluffy powder was highly water-soluble. Because of the variable amounts of volatile material usually present in lyophilized preparations, a sample was dried to constant weight at 100° at 0.2 mm before analysis and molecular weight determination. The loss in weight was 11.5%. The material was then no longer appreciably soluble in water. The free base of the peptide, which was obtained by passage of a solution of the acetate through an anion exchange column, Amberlite IR-45 in the hydroxyl form, followed by lyophilization of the effluent, also was not appreciably water-soluble. The dried material, like the free base, dissolved rather easily in 0.1 N acetic acid, however, and lyophilization of such a solution again yielded highly soluble material.

The dried material gave the following analysis: Calcd. for $C_{43}H_{66}O_{12}N_{12}S_2 \cdot C_2H_4O_2$: C, 50.6; H, 6.61; N, 15.8. Calcd. for $C_{43}H_{66}O_{12}N_{12}S_2$: C, 51.3; H, 6.61; N, 16.7. Found: C, 51.6; H, 6.77; N, 16.3 (determined on similar, separate sample).

Molecular weight determinations were carried out by use of a thermoelectric osmometer (5). Solutions approximately 0.05 molal with respect to sucrose in 0.1 M acetic acid served as standards. The values obtained on the original, lyophilized material and on the dried material were 969 (cor. for moisture) and 1220, respectively, which agree within the expected range with the theoretical values of 1068 and 1007 for the acetate and base, respectively, of isoglutamine-oxytocin. Derivation of molecular weight by this method, which is based on a colligative property, requires consideration of the number of particles/molecule afforded by the solute in solution. The calculated values are based on the assumption that the dried material provided one particle/molecule, but that the original acetate provided two.

A determination was also carried out after the dried material had been redissolved in dilute acetic acid and recovered by lyophilization of the solution. A value of 1108 (uncor.

for moisture) was derived for the molecular weight, based on the assumption that the material now provided 2 particles/molecule in solution. These results are therefore consistent with the other data which suggest that acetic acid is lost on drying. That perhaps other changes have also occurred in the process cannot be precluded by the present data.

Discussion. The foregoing data show that isoglutamine-oxytocin significantly inhibits the pressor activity of vasopressin administered to the anesthetized rat. The minimum effective dose under the experimental conditions described is approximately 50-60 γ /kg for 30 m μ of vasopressin. Because the pressor response to vasopressin is affected in the presence of anesthesia(6), these results cannot be extended directly to the unanesthetized animal without further investigation.

It may be recalled that vasopressin and oxytocin are closely related structurally, and this is manifested by the fact that many of the biological activities of the 2 hormones overlap. For example, vasopressin possesses some oxytocic activity, and oxytocin has a low degree of pressor activity(7). The isoglutamine isomer of oxytocin may therefore be considered structurally related to vasopressin as well as to oxytocin. This suggests that the inhibitory relationship that exists between vasopressin and isoglutamine-oxytocin may resemble in type many well established cases of inhibition based on the similarity in structure between the inhibitor and active compound. Despite the fact that the exact mode of action of hormones is not known, if this concept of inhibition does apply to the hormone under consideration, then one might picture in a general way the inactive isoglutamine compound as replacing vasopressin at the site of its action. Moreover, one might expect the corresponding isoglutamine isomer of vasopressin perhaps to inhibit vasopressin activity more effectively than does isoglutamine-oxytocin, since it would be closer in structure to the hormone than the latter.

The indication that acetic acid is lost from lyophilized preparations of isoglutamine-oxytocin acetate on rigorous drying is not without general precedence among salts of simpler bases. In the case of polypeptides and pro-

teins, however, it may have some special significance in relation to the phenomenon of inactivation. On drying solutions of the posterior pituitary hormones, as well as solutions of many enzymes, loss of activity is occasionally observed. It is well known that specific conditions of pH and charge favor maximum stability of these biologically active substances. The situation encountered with isoglutamine-oxytocin acetate suggests that, in general, a drying process may involve varying degrees of change in bound acid and charge, as well as in hydration, with the result that these biological substances may on occasion be left unexpectedly in a state less favorable with respect to stability.

Summary. 1) The isoglutamine isomer of oxytocin has been found to inhibit significantly the pressor activity of administered vasopressin in the anesthetized rat. 2) Various data suggest that acetic acid is lost when the polypeptide acetate is rigorously dried. Isoglutamine-oxytocin forms a crystalline flavianate salt.

Appreciation is expressed to Dr. V. du Vigneaud for his interest and suggestions. The authors would also like to thank Dr. H. B. van Dyke for his helpful discussion and Dr. W. D. Cash for his interest in connection with the assays. The elementary analyses were performed by Mr. Joseph Albert.

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Specific Function of Bile Salts in Cholesterol Absorption.* (23979)

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It has been demonstrated that bile salts are an obligatory requirement for passage of cholesterol from lumen of the intestine to the lymph(1). However, the mechanism by which bile salts facilitate absorption of cholesterol is not clear. Several explanations have been offered. One is that bile salts dissolve cholesterol by virtue of their hydrotropic action and that this process is facilitated when cholesterol is dissolved in fat(2). It has also been suggested that bile salts are necessary for cholesterol absorption because they function as co-factor for cholesterol esterase activity(3,4). Recently(5) it was demonstrated that a meta-

bolic pool of free cholesterol exists in the intestinal wall with which cholesterol from the lumen is mixed prior to its esterification and transfer to the lymph. The role which bile salts might have in the entrance of exogenous cholesterol-4-C¹⁴ into the metabolic pool of the intestinal mucosa and its subsequent transfer to the lymph was investigated.

Methods and materials. Rats with thoracic and bile duct fistulae were prepared and given saline to drink(5). Twenty-four hours after operation, each animal received by gastric intubation, 3 ml of aqueous emulsion containing cholesterol-4-C¹⁴ (0.5 μ C) alone or in combination with one or more of blood albumin, glucose, oleic acid, sodium dehydrocho-

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TABLE I. Cholesterol-4-C¹⁴ Recovered from Lymph and Intestinal Wall following Feeding of Cholesterol-4-C¹⁴ to Bile-Lymph Fistulae Rats.

Emulsion components*	Lymph		Intestinal wall	
	C ¹⁴ -free cholesterol	C ¹⁴ -esterified cholesterol	C ¹⁴ -free cholesterol	C ¹⁴ -esterified cholesterol
	% recovered			
3.3 mg cholesterol-4-C ¹⁴	.0	.0	.7	.0
2.3 " cholesterol-4-C ¹⁴	.0	.0	.4	.0
292 " oleic acid				
3.2 " cholesterol-4-C ¹⁴	.7	2.9	26.2	2.0
279 " sodium taurocholate				
3.0 " cholesterol-4-C ¹⁴	1.2	3.1	16.0	2.2
279 " sodium taurocholate				
292 " oleic acid				
2.8 " cholesterol-4-C ¹⁴	.0	.0	1.5	.0
279 " sodium dehydrocholate				
292 " oleic acid				

* Five animals/group, sacrificed at 6 hr; emulsion also contained 150 mg glucose, 50 mg albumin.

late and sodium taurocholate as indicated in Table I. Lymph was collected from 0-6 hours after administration of test meal and then animals were sacrificed. The small intestine was removed, washed and lipid extracts of lymph and small intestine were prepared as previously described(5). Free and total cholesterol were determined chemically as well as the C¹⁴-activity of the free, esterified, and total cholesterol of all extracts by methods described earlier(5).

Results. *Table I.* Following the feeding of a test meal containing approximately 3 mg cholesterol-4-C¹⁴ with or without oleic acid there was virtually no uptake of cholesterol-4-C¹⁴ by the intestinal wall and no transfer to the lymph. Addition of sodium taurocholate to the emulsion produced a large uptake of cholesterol-4-C¹⁴ (28.2%) by intestinal wall with almost all of the C¹⁴-activity in the free cholesterol fraction. There was also transfer of cholesterol-4-C¹⁴ to the lymph (3.6%), with a major portion of the recovered cholesterol-4-C¹⁴ in the esterified fraction. After feeding of a complete emulsion (cholesterol-4-C¹⁴, sodium taurocholate and oleic acid) there was considerable uptake of cholesterol-4-C¹⁴ by the intestinal wall and transfer to the lymph, but this uptake (18.2%) was less than when oleic acid was omitted from the emulsion. Feeding of a complete emulsion containing sodium dehydrocholate in place of sodium taurocholate

produced no uptake of cholesterol-4-C¹⁴ by the intestinal wall or transfer to lymph.

Discussion. There are several factors, namely, pancreatic cholesterol esterase, fatty acids, and bile salts which are required for transfer of exogenous cholesterol from the lumen to the free cholesterol pool of mucosa and then to the lymph. Cholesterol absorption does not occur in absence of bile or pancreatic juice, but will occur in absence of dietary fat; the fatty acids presumably can arise from endogenous sources(1,5-7). Pancreatic juice may be necessary due to its content of pancreatic cholesterol esterase which may be the enzyme involved in the esterification reaction prior to transfer of cholesterol from the pool in the mucosa to the lymph. Bile salts are required for this esterification reaction *in vitro*(3).

Our study provides evidence that one specific role of bile salts is that they are necessary for entrance of exogenous cholesterol into the mucosa. This process seems to require a specific type of bile salt since dehydrocholate was ineffective in promoting transfer of cholesterol-4-C¹⁴ to the mucosa or lymph. It is also apparent that if exogenous cholesterol cannot enter the free cholesterol pool of the mucosa it cannot be absorbed. In connection with these findings, it was previously demonstrated that dehydrocholate was inactive in catalyzing esterification of cholesterol with fatty acid by pancreatic cholesterol

esterase; also it did not produce a hypercholesterolemic effect when added to a high cholesterol diet(3,4). Both findings would suggest that a complex of cholesterol and bile salt is formed in the lumen which then enters the intestinal wall. It appears that entrance of free cholesterol into the intestinal wall does not depend on emulsification or solubility of cholesterol in fat droplets.

Summary. Entrance of cholesterol-4- C^{14} into the free cholesterol pool of mucosa and its subsequent transfer to the lymph requires the presence of a specific type of bile salt. This process does not require the presence of dietary fatty acid. It is suggested that free cholesterol in the lumen of the intestine com-

plexes with bile salt which then enters the free cholesterol pool of mucosa.

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Observations on Bacteriostatic and Bactericidal Action of Erythromycin.* (23980)

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Haight and Finland(1,2,3) have demonstrated that a growing culture of a sensitive strain of streptococcus is rapidly sterilized by erythromycin, whereas a fully grown culture of the same organism is insensitive to the bactericidal action of the drug. They conclude that "erythromycin may be either bacteriostatic or bactericidal depending on sensitivity of the organism and on concentration of the antibiotic. Erythromycin exerts its effect only against multiplying bacteria."

In the present work the effect of erythromycin on sensitive strains of *Staphylococcus aureus* was determined under various conditions. The results showed a striking effect of population density on bactericidal action of the drug.

Materials and methods. A strain of *S. aureus* very sensitive to erythromycin was kindly furnished by Dr. Lawrence Kunz of Mass. General Hospital. The organisms were

grown on nutrient agar slants and stored in a refrigerator. Log-phase cells were obtained by inoculating Trypticase Soy broth with small numbers of cells and incubating at 37°C until a concentration of approximately 10^8 organisms/ml was obtained (Klett reading = 30). Growth was followed either turbidimetrically with Klett Electrophotometer or by viable plate counts. For the latter, 0.1 ml samples of proper dilutions of culture were spread onto blood agar plates, read after 24 hours' incubation at 37°C. Purified crystalline erythromycin (980 μ g/mg) was the generous gift of Lilly Research Laboratories. Stock solutions containing 2000 μ g/ml of the drug were prepared in distilled water. These solutions were tested on pour plates and found to inhibit growth of organism at concentration of less than one μ g/ml. When measured by viable plate counts, the doubling times of cultures containing initially 10^3 and 10^8 organisms/ml were identical (26 min.). Furthermore, this same doubling time was obtained when growth of cultures containing 10^8 organisms/ml was followed turbidimetrically.

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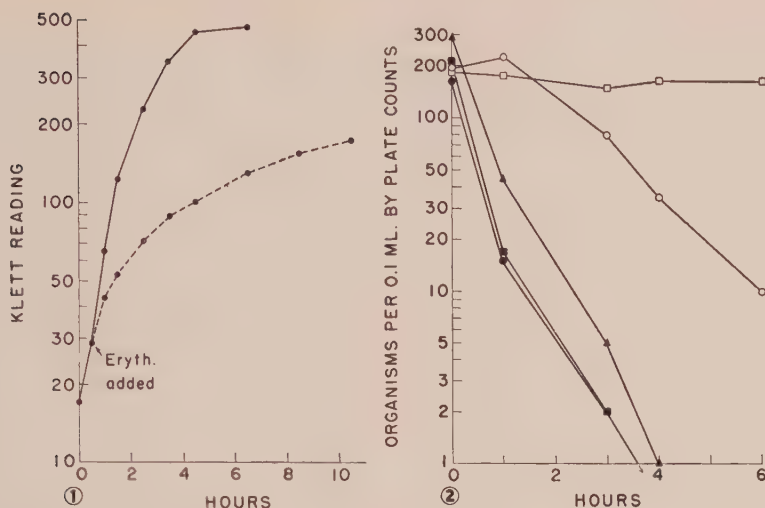


FIG. 1. Effect of erythromycin on growth of *S. aureus*. 50 ml cultures in 125 ml Erlenmeyer flask incubated with rotary shaking. 2 ml samples aseptically removed at intervals for turbidity determination. Erythromycin to make 4 µg/ml added as indicated. For other conditions see text.

FIG. 2. Effect of dense culture filtrate on action of erythromycin. Log-phase cells, diluted in warm broth to 10^8 organisms/ml were inoculated into media as follows: Broth ●—●; 1 part broth, 1 part saline ▲—▲; 1 part broth, 1 part drug-free culture filtrate ■—■; 1 part broth, 1 part filtrate with drug ○—○; organisms suspended in saline □—□. Erythromycin, 4 µg/ml. Each medium supplemented with 4 µg/ml of erythromycin at time of inoculation.

Results. Effects of erythromycin on growth of *S. aureus*: Fig. 1 shows results of experiment in which erythromycin (4 µg/ml) was added to one of a pair of flasks of growing bacteria, when the cell population in each had reached about 10^8 viable units/ml. The erythromycin permitted a striking further increase in turbidity, although at a rate that was significantly less than that of control culture. Similar results have been obtained with concentrations of the drug up to 200 µg/ml.

That cells responsible for this increase in turbidity were still sensitive to erythromycin was demonstrated by plating onto agar containing the drug. Growth was suppressed by concentration of 0.8 µg/ml.

In similar experiments it has been observed that increasing turbidity of the dense culture was paralleled by an increase in hemocytometer count, but no change in character of cells on Gram staining was detected. In contrast to above results, when the culture was diluted to 10^3 organisms/ml in broth, the drug was rapidly bactericidal (Table I).

Modification of action of erythromycin. To explain the difference in effects of erythromycin on heavy and light suspensions of bac-

teria, the culture fluid from a heavy suspension was examined. For this experiment cultures identical to those represented in Fig. 1 with and without erythromycin were prepared. After 24 hours' incubation the cultures were centrifuged and supernatants were sterilized by Seitz filtration. The pH of filtrates was 7.0. When filtrate from erythromycin-grown cells was added to an equal volume of broth containing erythromycin, the bactericidal action of the drug on low cell densities was depressed though not eliminated (Fig. 2). In contrast, the filtrate from cells grown without erythromycin had no effect. Dilution of broth with 0.85% saline resulted in a medium in which the bactericidal rate was

TABLE I. Effect of Erythromycin on Light and Heavy Cell Suspensions.

Hr	—Organisms/ml—		Klett reading
	Light cell suspension	Heavy cell suspension	
0	1810	181×10^6	30
3	10	218 "	91
10	0	130 "	162

At zero time a sample of heavy suspension was used to inoculate a second flask of medium at cell concentration indicated. For other conditions see text.

unchanged. In saline erythromycin exerted no killing effect.

Discussion. In our experiments visibly turbid cultures of *S. aureus* continued to grow in the presence of erythromycin at a concentration that was rapidly bactericidal to low-density log-phase cultures. Turbidity of culture increased from Klett reading 29 to 228. At the end of 24 hour incubation, microscopic examination of cells showed no change in size or shape, while hemocytometer counts showed increase in cell numbers. It is thus apparent that the increase in Klett reading was due to a net increase in number of cells in the suspension. This increase in cells, however, was not accompanied by a rise in number of viable units. Such results could be explained if growth and killing were proceeding simultaneously at rates that gave an approximately constant viable count. On the other hand, this discrepancy may be related to the data in Fig. 1 which on plain graph paper form a straight line. Thus, the increase in cell material was not attended by any net increase in synthetic ability. However, attempts to find this effect of the drug reflected in altered content of ribo- or desoxyribonucleic acid or protein in cells have been unsuccessful.

Pittenger *et al.* (4) reported that growth of *S. aureus* is significantly depressed by addition of erythromycin over most of the visible growth curve range. They found relatively low concentrations of the antibiotic to have a prompt inhibitory effect even when added to cells which reached the late log phase of growth. However, inhibition was progressively less complete as size of the inoculum increases. The present work is in agreement with that of Haight and Finland in showing erythromycin to be bactericidal to low density cultures. However, this paper also demonstrates that dense cultures of rapidly growing organisms are not subject to this bactericidal action. It is not clear whether the observations of Pittenger *et al.* demonstrate the same phenomenon, because they followed growth only turbidimetrically, and thus did not distinguish bacteriostatic from bactericidal action.

It would appear that only rapidly dividing organisms are killed by the drug. It might therefore be asked whether the protective action of the filtrate is due to some growth-inhibiting action on cells at low density. It can be seen in Fig. 2, by comparison with the medium diluted with saline, that mere dilution of medium by filtrate would not confer protection. Protection could occur if the filtrate contained a cytotoxic substance. Because the filtrate contains considerable erythromycin activity, this possibility cannot be tested with the same strain of organism. However, the filtrate supported rapid growth of a drug-resistant mutant derived from the same strain, using the filtrate as medium. Thus, a generally cytotoxic effect would appear to be ruled out.

This finding suggests that the protective action observed is most likely the result of decrease of growth rate by release of a specific factor, which appears to be released in the presence of erythromycin. It remains to elucidate its nature and the mechanism of its action.

Summary. 1. Erythromycin was bactericidal to low cell concentrations of growing *S. aureus* but not to heavy suspensions of the same growing cells. 2. Turbidity of the heavy suspension continued to increase after addition of erythromycin, although at a reduced rate. This increase was not paralleled by any increase in viable units. 3. Viable cells in a drug-treated suspension were still as sensitive to erythromycin as before addition of the drug. 4. Addition of a filtrate of a drug-treated, dense suspension to a dilute suspension of growing cells delayed onset and reduced rate of sterilization of these cells by erythromycin.

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Oxygen Uptake of Liver Slices from Rabbits Infected with *Eimeria stiedae*.^{*} (23981)

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Biochemical studies of host-parasite relationship using a protozoan parasite have been concerned with blood parasites such as *Plasmodium* and *Trypanosoma* for the most part (5). Nutritional relationship between *Eimeria neischulzi* and its host, the rat, has been extensively investigated with particular regard to vitamin content of host diet (1-4). *Eimeria stiedae*, the liver coccidium of rabbits, has received little attention from those interested in biochemical host-parasite relations. Smith and McShan (6) found that succinic dehydrogenase activity of rabbit liver homogenates was markedly increased after 10 and 15 days of *E. stiedae* infection, and decreased to a slightly elevated level by the twentieth day. They also found a decrease in percentage dry weight of infected liver, and interpreted this finding to be a result of liver edema.

This report deals with the oxygen uptake of infected liver slices incubated in a non-nutrient medium.

Material and methods. Crossbred white rabbits obtained from local dealer were used. These rabbits were examined for coccidiosis by fecal examinations before use. Animals between 2 and 5 months of age were employed. They were infected orally with a sublethal dose of sporulated oocysts. One-half of each litter was infected. An infected and a non-infected litter mate were sacrificed on third, sixth, tenth, thirteenth, seventeenth, twentieth and twenty-fourth days after infection. Three replications, consisting of 7 infected and 7 control animals, were made. Liver slices were prepared in Berkeley-type glove box, at low temperature (10-14°C). Each slice weighed approximately 100-150 mg (wet weight). Individual slices were suspended in Warburg flask containing 1.5 ml of pH 7.4 Krebs-Ringer phosphate buffer. The center well of flasks contained 0.15 ml of 20% potassium

hydroxide to absorb the CO₂. Duplicate flasks were prepared for each liver. Flasks were equilibrated in water bath at 38°C for 20 minutes. Readings were taken, thereafter, at 5 minute intervals for 30 minutes, and at 15 minute intervals for 2 hours. The QO₂ ww (oxygen uptake/mg wet weight/hr) was obtained by plotting cumulative microliters of oxygen used against time and drawing the best straight line for initial linear uptake. Pieces of liver tissue were dried in desiccator at room temperature over concentrated sulfuric acid to determine dry weight. Other pieces were analyzed for nitrogen by micro-Kjeldahl procedure. These data were used to calculate oxygen uptake/mg dry weight/hour (QO₂ dw) and oxygen uptake/mg N/hour (QO₂ n).

Results are summarized in Table I. Oxygen uptake in the infected livers was very significantly increased over that of non-infected controls ($P < .01$). This increased uptake became noticeable at 6th day, increased to a peak at 17th day, and declined slowly thereafter. Seventeen to 21 days after infection with *E. stiedae*, the oocysts make their first

TABLE I. Effect of *E. stiedae* Infection on Rabbit Liver.

	Days infected	QO ₂ dw*	% dry wt	QO ₂ n	% N
Control		1.35	29.3	17.60	13.89
Infected	3	1.48	30.6	23.09	15.72
Control		1.30	29.4	19.14	15.30
Infected	6	2.01	27.9	24.24	15.88
Control		1.37	30.3	18.86	13.88
Infected	10	2.04	26.2	24.34	13.76
Control		1.19	29.5	15.62	16.94
Infected	13	2.65	21.0	28.36	15.67
Control		1.80	26.9	18.56	17.31
Infected	17	3.92	20.7	36.28	16.01
Control		1.62	30.8	17.76	18.47
Infected	20	2.72	23.4	28.69	14.43
Control		2.23	27.0	22.40	15.37
Infected	24	3.97	20.4	32.25	14.96

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* Each value represents duplicate determinations in 3 replicates; thus, each figure is the mean of 6 determinations.

appearance outside the epithelial cells of bile ducts and there is a disappearance of schizogony. Further study is necessary to determine whether the increase in liver metabolic activity is due to a change in the parasite or whether the parasite change results from a metabolic alteration in the host. In a few experiments, the medium was enriched with adenosine triphosphate; this addition neither accentuated nor diminished the oxygen uptake.

Infection appeared to have little effect upon percentage of protein (nitrogen $\times$ 6.25) but percentage dry weight of infected livers was very significantly lower ($P < .01$) than that of the non-infected. This is in agreement with findings of Smith and McShan(6). These authors suggested that decrease in dry weight percentage was due to liver edema. Although total liver weights were not obtained in our experiments, an 8 to 9% decrease in dry weight (Table I) accompanied by 1 to 2% decrease in protein would imply either a considerable increase in total protein or a decrease of some other component. Since rabbit liver contains very little fat(7), glycogen depletion seems to be a logical explanation. Our oxygen uptake findings tend to confirm this possibility.

Summary. Oxygen uptake of liver slices

from rabbits infected with *Eimeria stiedae* was measured at various times after infection with a sublethal number of organisms. Oxygen uptakes of slices from non-infected littermates were determined concurrently. Measurements were made in a medium consisting of Krebs-Ringer phosphate buffer at pH 7.4 in a water bath at 38°C. Oxygen uptake of infected livers was significantly greater than that of non-infected controls. There was a significant decrease in percentage dry weight of infected livers while percentage protein was little changed.

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Differential Excretion of Cobalt-containing Products Following Administration of Co⁶⁰-labelled Vit. B₁₂ to Rats. (23982)

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Radioactivity excreted *via* the urinary tract after administration of Co⁶⁰-labelled Vit. B₁₂ has been demonstrated by microbial and radiometric(1), chromatographic(2), and extraction(3) studies to be the intact vitamin. While similar studies conducted on feces, following both oral and parenteral administration of radioactive vitamin, reveal a considerable amount of ionic Co⁶⁰, the results have been attributed(1) to microbial action on the

unabsorbed vitamin as it passes through the intestine.

Our data show that after parenteral administration of radioactive Vit. B₁₂ there is a differential excretion pattern between urine and feces that indicates alteration of the vitamin prior to its entry into the intestine. Neither sex nor testosterone propionate administration influences excretion of the radioactive substance.

Methods. A. Four male litters born to mothers maintained during pregnancy and lactation on a diet low in Vit. B₁₂, were used.

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From day of birth until 24 days of age, one-half of each litter (controls) received daily subcutaneous injections of 0.02 cc of cottonseed oil, whereas their littermates received a subcutaneous injection of 1 mg testosterone propionate† daily. From the 24th day of age until termination of experiment, controls received 0.06 cc of cottonseed oil and the testosterone-treated littermates received 3 mg of the androgen. All injections were administered with syringe controlled by a micrometer. When 30 days old, both groups received a subcutaneous injection of 0.25 μ g radioactive Vit. B₁₂.‡ The specific activity of this Cobalt⁶⁰-labelled preparation was 824 μ c/mg. Under the maintained injection technic and counting conditions, the total radioactivity administered was equivalent to 2,566 cpm $\pm$ 0.9%. Animals were weaned at 28 days of age and housed in individual cages. Immediately following administration of radioactive vitamin, each animal was placed in a similar metal cage to which absorbent paper had been affixed in such manner as to catch urine and feces. The wire-mesh bottom of the cage was silicined. The paper, containing the 24-hour urine sample, was changed daily. To avoid reciprocal radioactive contamination of excretion products, the feces were removed every 4 hours. Two of 4 litters received no further treatment following injection of tagged vitamin while the other 2 litters, beginning with the third day subsequent to radioactive injection, received a subcutaneous dose of 16.6 μ g stable Vit. B₁₂§ throughout the remainder of the 10-day collection period. Each absorbent paper was burned and quantitatively transferred to a counting planchet. To ascertain percent recovery of radioactivity by this method, 0.017 cc of Co⁶⁰-Vit. B₁₂ was delivered to a planchet containing 2 cc urine, while a similar preparation was transferred to absorbent paper. The former preparation was

dried; the latter was burned and quantitatively transferred to a planchet. Upon determination of radioactivity in each preparation, an average of 5 runs yielded 226 ± 2 cpm and 228 ± 2 cpm, respectively. Each 24-hour feces sample was counted separately from the urine sample. Radioactivity was determined with a scintillation detector (Nal crystal) encased in a lead shield and connected to a laboratory scaler. B. Individual 24-hour excretions of radioactivity in composite feces-urine samples of 7 females were compared with individual excretions of 8 males. To note whether there was an effect of exogenous androgen on either sex, the radioactive excretions of 7 androgen-treated females and 10 androgen-treated males were collected for comparison with radioactive excretions of control animals.

Results. A. Fecal excretion (Table I) following a subcutaneous injection of 0.25 μ g of tagged Vit. B₁₂ was low in the first 24-hour collection and increased to 3rd day, at which time there was a decrease in radioactive excretion. The urinary radioactivity was extremely high in the first 8-hour collection and then decreased markedly. Administration of unlabelled vitamin to animals previously injected with cobalt-tagged vitamin, was followed within 24 hours by approximately 6-fold increase in urinary radioactivity, above the level of animals not receiving the stable vitamin. An elevated level was maintained throughout additional 48-hour collection period. There was little or no effect on fecal excretion. The androgen (Table II) did not significantly affect either fecal or urinary radioactivity.

B. Sex had no significant effect on the 24-hour composite fecal-urine excretion of B₁₂ during various 24-hour periods (Table III). Likewise, androgen had no effect on radioactive B₁₂ excretion of either sex.

Discussion. The greatest urinary excretion of Vit. B₁₂, following its subcutaneous injection, occurs during the first 8 hours; the second 8-hour period contains less than 10% of the first period. This is, perhaps, attributable to the animal's lack of ability to retain the administered level of tagged vitamin. Chow *et al.*(4) noted a similar tendency in

† Testosterone propionate generously supplied by the Upjohn Co., Kalamazoo, Mich.

‡ We are grateful to Merck & Co., Inc., Rahway, N. J. for the generous supply of Co⁶⁰-labelled vit B₁₂.

§ The preparation employed was distilled water solution of 0.1% trituration of crystalline vit B₁₂ (Merck & Co.). As a preservative, phenol crystals were added to make 0.5% solution.

TABLE I. Control Rats. Excretion of radioactivity following parenteral administration of Co⁶⁰-labelled vit. B₁₂.

Time sequence	Pathway of excretion			
	Urine, cpm		Feces, cpm	
	Radioactive B ₁₂ only	16.6 γ non-labelled B ₁₂ daily from day 3	Radioactive B ₁₂ only	16.6 γ non-labelled B ₁₂ from day 3
0* - 8 hr	147 $\pm$ 10.2† (4)		8 $\pm$ 1.9 (12)	
8 - 16	12 $\pm$ 1.7 (4)		26 $\pm$ 4.0 (12)	
16 - 24	5 $\pm$.4 (4)		37 $\pm$ 5.5 (12)	
Total 1st day	174 $\pm$ 6.0 (12)		71 $\pm$ 5.4 (12)	
2nd	17 $\pm$ 1.1 (12)		110 $\pm$ 8.9 (12)	
3rd	13 $\pm$.3 (12)		155 $\pm$ 8.7 (12)	
4th	10 $\pm$ 1.3 (4)	66 $\pm$ 3.0 (8)	116 $\pm$ 15.9 (4)	125 $\pm$ 5.5 (8)
5th	10 $\pm$.8 (4)	36 $\pm$ 2.4 (8)	87 $\pm$ 7.7 (4)	102 $\pm$ 5.2 (8)
6th	9 $\pm$.5 (4)	21 $\pm$ 1.5 (8)	76 $\pm$ 9.7 (4)	53 $\pm$ 2.8 (8)
7th	9 $\pm$.3 (4)		54 $\pm$ 2.8 (4)	
8th	8 $\pm$.0 (4)		43 $\pm$ 1.9 (4)	
9th	8 $\pm$.3 (4)		33 $\pm$ 3.3 (4)	
10th	7 $\pm$.0 (2)		23 $\pm$.5 (2)	
Total excr. % of inj. dose	10.3		29.9	

* Time of inj. of labelled vit. B₁₂ is referred to as "zero" time. † Stand. error of mean.
 Figures in parentheses represent No. of animals.

humans. Although subsequently recorded urinary radioactivity was low, the fecal radioactive count increased until approximately the end of the third day, then steadily decreased. Table I shows that, at all collection points past the first 8-hour period, fecal radioactivity was many times that of the relatively insignificant radioactivity in urine. Then, it ap-

pears that a plentiful supply of Co⁶⁰-labelled substance is available for excretion both during the first 8-hour period and any subsequent first, second, or third-day period. But it is also apparent that during the first collection period, radioactivity is excreted mainly in the urine, while during any subsequent period fecal excretion predominates. Thus we can sur-

TABLE II. Testosterone-Treated Rats. Excretion of radioactivity following parenteral Administration of Co⁶⁰-labelled vit. B₁₂.

Time sequence	Pathway of excretion			
	Urine, cpm		Feces, cpm	
	Radioactive B ₁₂ only	16.6 γ non-labelled B ₁₂ daily from day 3	Radioactive B ₁₂ only	16.6 γ non-labelled B ₁₂ from day 3
0* - 8 hr	164 $\pm$ 5.8† (4)		8 $\pm$ 1.9 (12)	
8 - 16	11 $\pm$.9 (4)		32 $\pm$ 3.5 (12)	
16 - 24	8 $\pm$.6 (4)		33 $\pm$ 4.8 (12)	
Total 1st day	161 $\pm$ 6.6 (12)		73 $\pm$ 4.8 (12)	
2nd	19 $\pm$ 1.7 (8)		114 $\pm$ 5.2 (12)	
3rd	11 $\pm$ 1.1 (8)		153 $\pm$ 5.8 (12)	
4th	11 $\pm$.2 (4)	61 $\pm$ 2.8 (8)	136 $\pm$ 7.9 (4)	158 $\pm$ 10.2 (8)
5th	10 $\pm$.2 (4)	34 $\pm$ 1.6 (8)	84 $\pm$ 4.5 (4)	99 $\pm$ 8.3 (8)
6th	9 $\pm$.6 (4)	21 $\pm$.4 (8)	62 $\pm$ 4.6 (4)	59 $\pm$ 5.4 (8)
7th	9 $\pm$.6 (4)		55 $\pm$ 5.0 (4)	
8th	8 $\pm$.3 (4)		50 $\pm$ 5.6 (4)	
9th	7 $\pm$.0 (4)		33 $\pm$ 4.8 (4)	
10th	7 $\pm$.4 (4)		23 $\pm$ 1.6 (4)	
Total excr. % of inj. dose	9.8		30.5	

* Time of inj. of labelled vit. B₁₂ is referred to as "zero" time. † Stand. error of mean.
 Figures in parentheses represent No. of animals.

TABLE III. Total Radioactive Excretion* of Female and Male Rat following Parenteral Administration of Co⁶⁰-labelled Vit. B₁₂.

Time sequence	Control rats		Testosterone-treated rats	
	Female (7)	Male (8)	Female (7)	Male (10)
1st day	266 ± 18.4†	283 ± 23.5	289 ± 29.8	270 ± 29.2
2nd	162 ± 24.1	152 ± 19.3	169 ± 13.9	164 ± 14.8
3rd	165 ± 14.6	157 ± 17.1	180 ± 9.3	166 ± 17.8
Further treatment	Non-labelled vit. B ₁₂ , subcut., daily—			
4th day	215 ± 9.4	223 ± 16.2	228 ± 15.5	219 ± 9.3
5th	159 ± 10.2	154 ± 7.7	161 ± 8.2	154 ± 6.3
6th	124 ± 9.3	139 ± 16.8	118 ± 9.9	115 ± 5.1

* Avg values for composite urine-feces samples are reported as cpm.

† Stand. error of mean.

mise that there are at least 2 different Co⁶⁰-labelled substances, one excreted by the urinary tract, the other by the intestinal tract; whether these routes of exit are decidedly exclusive cannot be ascertained from the data on hand. The feces-destined substance appears to be an altered one, since its elimination continues to increase to the end of the third day. The tagged urine molecule may be the intact vitamin or a close associate of it since: 1) it rapidly appears in urine, and 2) upon a large injection of non-radioactive preparation of Vit. B₁₂, urinary excretion of radioactivity is increased approximately 5 times above its preceding level, whereas fecal radioactivity is not appreciably altered. Microbiological assays(1), extraction(3), and chromatography(2), appear to eliminate the possibility that tagged urinary substance might be inorganic cobalt.

The explanation for the interesting observation, that fecal radioactivity progressively increases (rather than decreases) past the first day until the fall noted upon examination of the 4th day collection remains at present unanswered. These results are at variance with Barbee's and Johnson's data(2) which show a decrease in fecal radioactivity subsequent to the first day's administration of radiovitamin. The disparity may, perhaps, be accounted for by difference in dosage level used.

Becker, Lang, and Chow(5) found that although radioactive Vit. B₁₂ content was decreased in liver and kidney, the urinary radioactivity following testosterone administration was not significantly altered. Our data show no effect of androgen on either urinary or fecal excretion patterns.

Summary. 1) The excretion pattern of radioactive substance following parenteral administration of 0.25 µg of Cobalt⁶⁰-labelled Vit. B₁₂ to the young rat has been studied. 2) Urinary excretion of radioactivity reaches a maximum during the first 8 hours after labelled Vit. B₁₂ administration, then rapidly declines; fecal excretion rises progressively through the 3rd day. Moreover, administration of a large dose of unlabelled Vit. B₁₂ subsequent to radioactive B₁₂ significantly increases urinary but not fecal excretion of radioactivity. From this differential excretion pattern, it is inferred that labelled vitamin is altered prior to its excretion into the intestinal tract. 3) The fecal route is the major excretory pathway for radioactivity resultant from parenteral introduction of Co⁶⁰-labelled Vit. B₁₂; after the 1st 8 hours, the ratio of radioactivity excreted for a 10-day period *via* fecal and urinary pathway is 7:1, respectively. 4) Sex does not appear to influence excretion of Co⁶⁰-tagged products. Similarly, administration of large quantities of testosterone propionate to either females or males does not alter the excretion pattern.

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Influence of Histamine Liberator Substance 48/80 on Basophil Leucocytes of Rabbit Blood.* (23983)

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Different views about the functional relationship of basophil leucocytes and tissue mast cells have been published. Ehrich(3) and Asboe-Hansen and Kaalund-Jørgensen (1) expressed the view that blood and tissue mast cells may have some functional similarities. Mast cells(6) and basophil leucocytes (4) have been reported to contain histamine. A chemical histamine liberator of low toxicity, compound 48/80, which is a polymeric condensation product of p-methoxyphenethyl-methylamine and formaldehyde, seems to bring about histamine release from tissue mast cells(7). This report concerns the effect of compound 48/80 on basophil leucocytes of rabbit blood.

Methods. Twenty-eight white male rabbits, of body weight between 2500 and 3000 g were used. The animals were kept in separate until they became accustomed to handling and blood sampling. Compound 48/80[‡] in physiological saline was injected intravenously 1 mg/kg body weight. Injection was performed slowly in the marginal ear vein at about 9 a.m. Blood samples for basophil and total leucocyte counting were obtained from the ear veins just before injection as well as after 2, 4, 6, and 24 hours. Control counts were obtained from the same animals, getting corresponding volumes of physiological saline injected on a day within the previous week. A modified Moore and James diluting fluid(5) was used for direct basophil counting, consisting of: .05% toluidine blue in a phosphate buffer (pH 7.7) 40.0 ml; 96% ethanol, 5.5 ml; ammonium-sodium oxalate anticoagulant mixture, .5 ml. After mixing

well, the fluid was filtered, and to it was added 0.5 ml of a saponin solution in 50% methanol (280 mg saponin in 10 ml alcohol). The fluid was filtered each time before use. Blood diluted 1:10 and Fuchs-Rosenthal counting chambers were used. Relative basophil counts (number of basophils/100 leucocytes) were obtained.

Results. All animals initially developed symptoms of distress such as prostration and difficult respiration, but with varying severity. Within half hour the animals appeared almost normal again.

The majority of treated animals demonstrated a fall in basophil counts compared to control counts (Fig. 1). Six hours after injection of compound 48/80 a decrease was observed in 23 animals; even after 24 hours 19 animals failed to regain their pretreatment levels.

The maximum drop in number of basophil leucocytes appears to take place 6 hours after injection of compound 48/80 (Statistical significance: $P < 0.01$) (Fig. 1, Table I).

Discussion. Riley and West(6,7) reported that the histamine content of tissue mast cells may be released as a response to administration of different histamine liberators, including compound 48/80. They assumed a binding of histamine to the granular mucopolysaccharide content of mast cells, and thought that a granule release was the morphologic picture of the histamine release(7).

Asboe-Hansen and Wegelius(2) studied the effect of histamine liberators including com-

TABLE I. Change in Relative Basophil Counts (Avg for 28 Rabbits) following a Single Intrav. Inj. of Compound 48/80 (1 mg/kg).

Initial		6 hr after inj.	
Control	Exp.	Control (saline)	Exp. (48/80)
3.44	3.52	5.05	2.88
Statistical significance: $P < .01$			

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[‡] Kindly supplied by Burroughs Wellcome & Co., England.

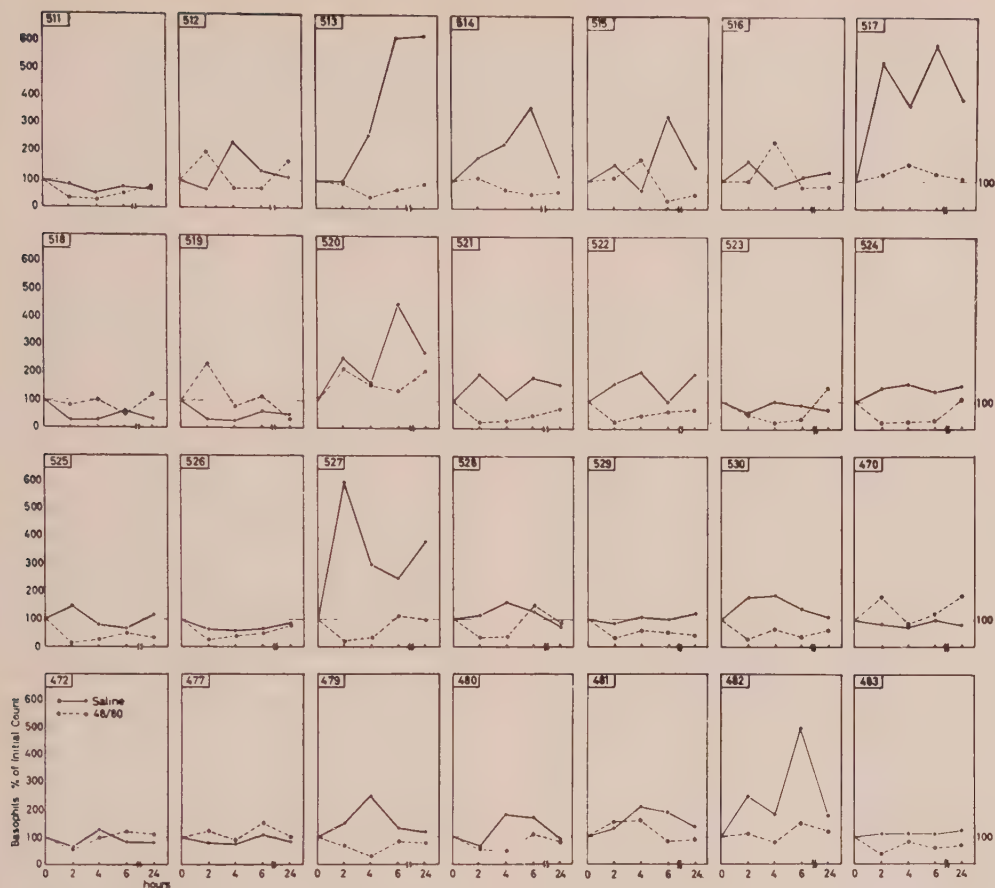


FIG. 1. Changes in relative counts of circulating basophil leucocytes in rabbits following a single intrav. inj. of 1 mg/kg of compound 48/80 (●—●). Control counts (○—○) were obtained, following inj. of a corresponding vol of saline in the same animal, on a previous day.

pound 48/80, on living connective tissue of the hamster cheek pouch. These authors reported a degranulation of living mast cells following systemic administration of compound 48/80. They interpreted the degranulation as a non-specific response to an increase in tissue water due to increased capillary permeability provoked by histamine liberated from some source. They suggested that the mucopolysaccharide originating from mast cells may bind water and thus convert the extracellular water to a ground-substance like hydrated gel.

If the histamine liberator injected has a direct action on basophils, these may release their metachromatic granules and thus escape detection; the basophil count consequently falls.

In the rabbit connective tissue mast cells are comparatively few, while the number of circulating basophils is relatively high. So, if a state of tissue oedema due to histamine liberation occurs as a response to administration of compound 48/80, and the few tissue mast cells are not capable of coping efficiently with such an emergency, the basophil leucocytes might migrate from the circulation to the oedematous tissues substituting their function of organizing the tissue oedema.

Summary. A decrease in number of circulating basophil leucocytes in rabbits occurred within 6 hours after injection of the histamine-liberator substance 48/80. The possible relationship between basophil leucocytes and connective tissue mast cells and especially a supplementary function of mucopolysacch-

aride release is discussed.

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Etiology of Chemically Induced Writhing in Mouse and Rat. (23984)

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Since it is generally agreed that analgesic compounds aside from opiates have little effect when tested by technics involving infliction of thermal or mechanical pain in normal experimental animals, many investigators have searched for methods which assess activity of "low potency" analgesics. Among these are Vander Wende and Margolin(9), and Siegmund *et al.*(7). The former group employed sodium iodomethamate intraperitoneally to produce "writhing" (a syndrome consisting of intermittent rubbing of the belly on the cage floor) in rats and arrived at oral PD_{50} values for opiates and antipyretic analgesics. This group blocked writhing by previous injection of procaine intraperitoneally. Siegmund *et al.* employed mice in a similar study using phenylquinone as inciting agent for writhing. 5-Hydroxytryptamine (5-HT) produces severe pain on intradermal injection (Armstrong *et al.*(1)) and has been reported (Lu, personal communication) to produce writhing when injected intraperitoneally in mice.

We studied effects of morphine, aminopyrine, lysergic acid diethylamide (LSD) and chlorpheniramine in prevention of writhing by phenylquinone, 5-HT and other substances, to arrive at the mechanism whereby writhing is produced and to ascertain optimal inciting agent of those mentioned above for efficient testing of new compounds.

Methods. Male Carworth mice weighing 18-22 g were distributed into groups of 5 animals each. Compounds under considera-

tion as writhing producing agents were injected intraperitoneally, in aqueous solutions. Compounds investigated as blockers of writhing were administered subcutaneously, one-half hour prior to injection of the writhing agent. One group received only the writhing agent and served as control in each experiment. The usual period of observation for presence or absence of writhing was 10 minutes. If no response was observed the material was considered ineffective. If writhing was observed after this period, it was classified as "delayed." Such results are marked with an asterisk in the Table which gives number of animals which writhed, divided by total number in the group. Rats were also employed, weighing 150-200 g and treated in the same manner. Phenylquinone, histamine, which is commonly believed to play a role in inflammation, serotonin, heparin, and various releasers of these substances (Table I) such as L-1935, 48/80, and reserpine were used as writhing-inciters. NaOH and HCl were employed to produce pain, possibly without specific release of other substances. Distilled water and 0.9% NaCl solution were also tried. Writhing inciters were given in total volume of 0.25 ml/mouse or 1 ml/rat. Since our primary object was to attempt to elucidate the mechanism of action, we chose 4 drugs with different pharmacological activities, as possible blockers of the writhing effect: Morphine (a narcotic analgesic), aminopyrine (a non-narcotic analgesic), lysergic acid diethylamide (a blocker of serotonin), and chlorphe-

TABLE I. Substances Released by Various Compounds.

Compound	Substance released	Reference
48/80	Histamine-partial (mouse)	Riley and West(6)
"	5-HT and histamine (rat)	Bhattacharya and Lewis(2)
5-Hydroxytryptamine	Histamine (rat)	Sparrow and Wilhelm(8)
Histamine	No release of 5-HT in rat	Bhattacharya and Lewis(2)
L-1935	Histamine (rat)	Feldberg and Lecomte(3)
Reserpine	5-Hydroxytryptamine (rat)	Parratt and West(4)
48/80	Heparin (rat)	Riley, Shepherd, West, Stroud(5)

niramine (a potent anti-histamine). The results are summarized in Table II.

Results. Phenylquinone, as well as 5-HT, L-1935 and dilute hydrochloric acid produced immediate writhing in the mouse, differing only in duration. Writhing persisted for more than one hour after phenylquinone, but only 20-25 minutes after other inciters. Such agents as heparin, reserpine, water and saline produced delayed writhing whereas dilute sodium hydroxide and 48/80 gave no response. Histamine and bradykinin (not tabulated) were variable in effect.

In the rat, phenylquinone and HCl were the only substances of this group which induced writhing.

Among the various possibilities as to etiology of writhing appeared the following: 1. Direct irritation; 2. Release of serotonin; 3. Histamine release.

The pH values of solutions producing writhing, ranged between 4 and 5. If direct irritation by acidity of drug were causative in writhing, any non-acid substance should be

inactive. To test this theory, we first injected a solution of serotonin at pH 4.7 which gave a 100% response, then neutralized the solution to pH 7 and tested it again on another set of mice. All of these mice also writhed. This result appears to rule out acidity of inciter as the only causative agent. Sodium hydroxide did not produce writhing in either species so that an elevated pH appears not to be concerned in the phenomenon. Reserpine, a serotonin releaser in the rat, produced only delayed writhing in the mouse and was inactive in the rat. Compound 48/80, known to release serotonin in the rat, did not produce writhing in either species at doses from 1 to 2.5 mg/kg. Serotonin itself produced writhing only in the mouse. These findings suggest that serotonin is not primarily etiologic.

Although serotonin releases histamine in the rat the reverse is not the case. Histamine itself was only variably active as a writhing inciter in the mouse and not at all in the rat. The anti-histamine chlorpheniramine produced no decrease in percentage of mice which

TABLE II. Blockade of Irritant Induced Writhing.

Irritant	Dosage, mg/kg	—		Aminopyrine, 100 mg/kg		Morphine, 1.0 mg/kg		LSD, 5.0 mg/kg		Chlorpheni- ramine, 5 mg/kg	
		Mouse	Rat	Mouse	Rat	Mouse	Rat	Mouse	Rat	Mouse	Rat
Phenylquinone	2.5	50/50	5/5	0/5	4/5	0/5	4/5	5/5	2/5	5/5	4/5*
Serotonin	"	32/35	0/5	"	"	"	"	1/5	"	3/5*	"
Histamine	"	15/25*	"	"	"	"	"	"	"	8/15*	"
Hydrochl'c acid	10.0	15/15	5/5	"	2/7	"	0/5	4/5	0/5	5/5	3/5
L 1935	2.5	10/10	0/5	"	"	"	"	0/5	"	2/5*	"
Reserpine	"	1/15*	"	"	"	"	"	"	"	3/5*	"
Sodium hydroxide	"	0/5	"	"	"	"	"	"	"	"	"
48/80	"	0/10	"	"	"	"	"	"	"	"	"
Heparin	"	0/5*	"	"	"	"	"	"	"	"	"
Water	"	"	"	"	"	"	"	"	"	"	"
Saline	"	"	"	"	"	"	"	"	"	"	"

* Plus some animals which showed delayed writhing.

Above figures signify $\frac{\text{No. of animals writhing}}{\text{No. of animals tested}}$.

writhed after histamine. The histamine releaser I-1935 produced immediate writhing in the mouse but its action was only partially blocked by chlorpheniramine during 10 minutes of observation. Compound 48/80, also a histamine releaser in the rat was inactive in the mouse. These observations are not consistent with the hypothesis that histamine release (as judged by data on such release in rats to be sure) may be an inciting cause of writhing in the mouse. However, it is apparently unknown whether L-1935 can release substances other than histamine. Compound 48/80 was only partially effective as histamine releaser in the mouse(6).

Since phenylquinone and hydrochloric acid were the only effective writhing producers in the rat, it would seem that release of serotonin or histamine is not to be considered in causation in this species.

Writhing in the mouse was blocked in all cases by morphine, LSD, and aminopyrine except that LSD did not block writhing produced by phenylquinone or hydrochloric acid. Chlorpheniramine partially blocked writhing produced by histamine, serotonin, or L-1935. It did not block writhing produced by phenylquinone or hydrochloric acid.

Summary. 1. Histamine is probably not a basic cause of writhing in the mouse. 2. It is possible that 5-HT may be a basic etiologic agent but, if true, the usual releasers of 5-HT do not so function in the mouse. 3. Acidity

of injected solution *per se* is probably not etiologic. 4. It is worth considering that phenylquinone, HCl, serotonin, and L-1935 may release some substance other than histamine, serotonin, heparin, or bradykinin which in turn may incite writhing. 5. Direct irritation of nerve endings remains as a distinct causative possibility. 6. In screening for analgesics, both opiate and non-opiate types, phenylquinone and HCl are most useful of writhing agents tried, in eliminating the possibility of false positives. Phenylquinone may be preferable because of its greater duration of action.

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Determination of Cell Viability. (23985)

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The difficulties which arise from attempts to assess viability of tissue cells solely in terms of reproductive capacity include: suitability of substrates and circumstance; necessity of employing population data to deduce what has happened to individuals; ultimate impossibility of distinguishing "static" from "cidal"; and the fact that certain types of tissue cells have not been induced to prolifer-

ate *in vitro*. Ideally, viability should be determined by methods which are simple, rapid, and applicable to the non-proliferating individual as well as to cultivable populations. If diverse factors required for reproduction are set aside, the capacity to control internal ionic environment is a universal characteristic of living beings. Many observations concerning exclusion of dyes by living yeasts(1,2), bac-

TABLE I. Reagents and Materials.

Bacto-Eosin Y (DE-5): 1.0% in H ₂ O			
<i>Idem</i> : 0.15% in BSS			
Levy-Hausser hemocytometer			
Clean glass slides, 1 × 3"			
Standard platinum loops:			
Diam., mm	AHT* No.	Vol† de- livered, ml	Approx. vol ratios
4	7433B	.01750	80
2	7433	.00250	10
1	7433	.00038	2
.7	8304-G	.00022	1

* Arthur H. Thomas.

† When filled by lifting horizontally through surface of liquids. If withdrawn vertically, the loops transfer approximately ½ the stated volumes.

teria(3) and tissue cells(4,5,6) have readily been confirmed. In view of materials, time and attention required to assess viability of tissue cells and cell cultures by growth determinations, and the ultimate uncertainties of interpretation, it seems useful to emphasize and illustrate the convenience with which the more fundamental criterion of dye exclusion may be applied.

Methods. Useful solutions and materials are indicated in Table I. The 1% stock solutions of eosin are self-sterilizing, or may be autoclaved in water or balanced salt solution (BSS). Since known, reproducible volumes can be transferred by platinum loops, these are used to measure solutions and cell aliquots. If stock solutions are prepared at appropriate concentrations, any desired combinations or dilutions can be prepared in droplets on glass slides. Also, small loops of a reagent may be mixed with the contents of a larger loop, and aliquots dispensed from the latter. Transfer of alkali or metal residues is prevented by swishing the loops in water before drying or flame sterilization. Dilution of cells in dye solutions prior to hemocytometer counts is obviated by drying 1mm loops of aqueous eosin 1% on the inclines to the chamber, and by use of a stippling motion to dissolve the dye uniformly in a 2mm loop of cell suspension before sweeping the mixtures into the counting chambers. As a general method it is more convenient to store clean slides on which 1mm loop-spots of aqueous eosin 1% have been dried (usually 3 horizontal rows of 5 spots each). During tests, a slide is laid

in a Petri dish containing (in both the top and bottom) 90mm circles of moistened filter paper. One or more eosin spots are rehydrated with 2mm loops of cell suspension, stirring or stippling to ensure prompt mixture. In the case of dense cell suspensions, the eosin is rehydrated with a 2mm loop of test solution or serum 2% in BSS (without bicarbonate), and a wire or a 0.7mm loop of cells is added. The slide is held in the moist chamber at room temperature for 2 minutes before observations on 200 cells/spot are recorded. If care is taken to transfer uniform aliquots and cells are not too numerous, it is also practical to enumerate total cells per droplet. Nicely stained cells are seen in air-dried droplets which have been extracted for 2 minutes in 95% alcohol or in 10% formalin, rinsed in water, and again dried.

Addition of small loops of test solutions to 2mm loop-droplets of cells in the absence of eosin will be recognized as a means of imposing experimental variables. If counts are not to be made immediately, the droplets are prepared with minimal spreading. The slide is held in a moist Petri dish or in a horizontally placed Coplin jar into which CO₂ can be exhaled and trapped. While the 1mm loop of eosin 1% is being added to assess the results, the droplets are spread to diameters of 6-7mm. For direct observation of viability among cells growing on glass in liquid media, it is simplest to remove the medium to 0.1 ml, to add one 4mm loop of eosin 1%, and to wash this solution over the cells for 2 minutes. After gross or microscopic observation, the cultures are extracted twice for 5 minutes with BSS before adding the renewal medium. Most observations have been made on clone 929 of Earle's L strain of mouse fibrocytes derived from a medium comprised of horse serum 2%, Eagle's supplement(7) and Bacto-peptone 0.5% as recommended by Waymouth(8). If experimental background is not stated, these and other cells were suspended in serum 2% in BSS without added bicarbonate during tests for dye exclusion.

Results. The simplest evidence that eosin distinguishes live from dead cells is as follows. Cells which exclude eosin in moist preparations are seen to be plump and smooth.

TABLE II. Effect of Variable Serum Concentrations.

Counts at	% cells white			
	Serum: .04%*	.2%	2%	20%
2'	92	90	90	96
15'	85p	95	90	94
30'	54p	90	92	90
Avg % white		92	91	93

Continuous exposure to 0.15% eosin in loop spots on 3 slides in moist Petri dishes.

One slide examined at each interval.

* Concentration of serum in "BSS controls."

p = cells acquiring pink tinge of eosin.

Those which admit dye resemble flattened spheroids. With appropriate illumination, they exhibit the roughened surfaces which always are seen in motion photomicrographs of cells which undergo a fantastic boiling and bubbling just as they expire. Since these death throes of the cell are as well known, and as readily interpreted as the failure of heart beat or respiration in larger animals, the association between eosin staining and death of the cell seems to be a direct one.

Major variables which might influence the validity of methods based on the percentages of cells capable of dye exclusion are: serum concentration, dye concentration, and time of exposure before counting. Because of inevitable pH drift and dehydration, these factors might be expected to cause difficulties in small droplets on glass slides.

As shown in Table II, simple assessments of viability of the L cells (counts within 2-5 minutes) were not modified by a 500-fold range of serum concentrations. Results remained constant during exposure to 100-fold variations in serum background for 30 minutes. Since yeasts are not dependent on serum for maintenance, their use permitted further clarification of this question. Following 5 hours of continuous exposure to 0.15% eosin in serum 0% and 20%, the percentages of white cells were 86% and 88% respectively. Thus, no evidence was obtained that serum binding of eosin alters its availability at pH values above 7.

The results in Table III demonstrate that in the presence of serum 2% these cells tolerated twice the recommended concentration of eosin for 30 minutes and that 30 minute

exposures usually were required to demonstrate the deleterious effect of 3 times the recommended concentration.

The tolerance of cells to dehydration of small droplets during counting on glass slides is explained by their adaptability to a wide range of osmotic pressures. Dilute suspensions of the L cell in serum 0.4% and 2% were exposed to modified electrolyte concentrations for periods of 1 hour; 2mm loop samples were assayed on eosin slides at 15, 30 and 60 min. to determine the onset of damage; the cells were then washed and replanted in the original tubes to confirm the number and quality of surviving cells. Within less than 3 minutes cells in BSS 200% became much smaller than usual, while those in BSS 20% and 10% were expanded to many times their normal size. Both eosin and cultivation revealed that 200% to 20% of the usual electrolyte concentration was tolerated for 1 hour; also that the onset and degree of damage in BSS 10% water 90% varied with the quality of cells employed in different experiments. In the presence of BSS 10%, the earliest indications of the onset of damage were revealed by the fact that 90-97% of cells excluded eosin for 2 minutes, but were unable to exclude it for 10 minutes.

The practicality of employing dye exclusion to replace cultivation procedures is now to be illustrated by a few examples.

The merits and liabilities of the two customary methods of preparing cell suspensions—scraping or enzyme release—were readily defined by direct observation in loop-spots on slides or by sampling from tubed suspensions which permitted more accurate control of pH

TABLE III. Effect of Increased Eosin Concentrations.

Counts at	% cells white			
	Eosin: —*	.15%	.30%	.45%
2'	88	91	93	90
15'	93	90	92	88
30'	88	87	89	87p
Avg % white	90	90	91	(89)†

Continuous exposure as in Table II: Serum 2%, eosin variable.

* Control: Eosin added just 2' prior to each examination.

† Avg after 2' and 15' exposure.

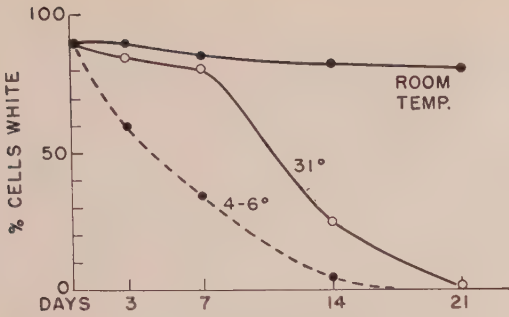


FIG. 1. Viability of dilute suspensions of L cells during storage without pH adjustment or renewal of medium. Cells: $530 \times 10^3/\text{ml}$. Fluid depth: 8 mm. Assays = transfer of 2 mm loops of cell suspension to eosin slides; counts after 2 min.

and evaporation. When approximately half the cells were removed from 5 culture tubes by firm strokes with a rubber policeman the percentages of cells excluding eosin were: 69, 88, 75, 73 and 89% (average 79%). Vigorous, 3x aspiration of these suspensions lowered the average values by 10%. The remaining cells, removed by pancreatin 0.1%,* revealed uniformly high viability, the average value being 96%. Excessive pancreatin concentrations or exposure times, however, were unfavorable. During exposure of suspensions containing some 20% dead cells to pancreatin 0.3% or 0.9% for 30 minutes, the percentages of viable cells rose to 95%, while counts of total cells dropped.

The data in Fig. 1 represent determinations of optimal conditions for the storage of stock cell suspensions in siliconed tubes. It is evident that the simplest alternative (to let the culture tubes stand in a block on the bench) assures useful suspensions over a period of three weeks. In other experiments, it was shown that periodic adjustment of pH improved maintenance at 31°.

The direct simplicity of identifying favorable or unfavorable circumstances for cells residing on glass is emphasized, particularly because observations on dye exclusion can be made *in situ* and without interference with the continuation of the cultures. If supernates are withdrawn to 0.1 ml and a 4mm loop of eosin 1% is added, macroscopic observation reveals within one or 2 minutes any portions of a

cell sheet which have been damaged. This permits removal of such areas while rinsing out the eosin in preparation for renewal, and spares viable portions of the culture from contending with dyed cells and their debris. Culture tubes of FJ cells were compared after standing upright for one hour at room temperature, some with tight caps and others improperly sealed. Microscopic examination of the sealed tubes revealed a few dead cells near the upper margins of the culture, while inspection of the others showed that death had occurred halfway downward through the cell populations.

Though these methods have not been used in a systematic study of the way in which tuberculin initiates damage to sensitive cells, it may be noted that the addition of eosin to cultures of spleen cells from normal and tuberculin sensitive animals revealed the specific damage of sensitive cells by OT within 24 hours, and before direct microscopic examination permitted decision.[†] Rinsing and renewal of cultures revealed that eosin did not interfere with continued maintenance of the relatively delicate cell types.

Identification of cells subject to physiological deficits. Whether good cells or poor ones are examined within a few minutes after the addition of eosin, differentiation of living from dead cells is simple and decisive. The cells are either white or red. Examination of the more shadowy hinterland of cells which are exposed to eosin while embarrassed in various ways teaches two lessons: (a) the desirability of standardizing serum background and routine working conditions and (b) the possibility that capacity for dye exclusion might replace more time-consuming methods in the study of depleted cells and their nutritional or metabolic requirements. A general state of deficiency in suspensions or cultures which contain a high incidence of viable cells on initial counts is revealed by the fact that almost this exact proportion of cells soon acquires a pink tinge of eosin within the cytoplasm (Tables II and III). Such deficiencies have been observed in cells from cultures which had not been renewed at date, in cells refrigerated

* Difco Pangestin 1:75, Control #419 221.

† Observations with Dr. Marcus Brooke.

several hours in dilute pancreatin and serum digest, and in cells exposed to rigorous washing. If such cells are observed during continuous eosin exposure, there are readily revealed: a greater susceptibility to increased concentrations of eosin, and the beneficial effects of brief prior incubation in serum or a nutritional medium.

Discussion. While theoretical soundness and universal applicability are prime qualifications of the principle of dye exclusion, practicality may determine its general usefulness. Small numbers of cells suffice for microscopic determinations of viability. Replacement of sterile tubes and pipettes with standard loops, counting in eosin-containing droplets on ordinary slides, and direct observation in cell cultures *in situ* facilitate application of this principle to cell suspensions and cultures. The relative non-toxicity of eosin is revealed by the tolerance of cells to unnecessary concentrations of dye and by continuation of epithelial cell, L cell and spleen cell cultures after the recommended exposures. Pappenheimer(5) has stated that the concentrations of trypan blue required for reproducible estimates are roughly proportional to serum concentrations. It is probably an advantage of eosin that minimal binding by proteins occurs in the physiological range of pH values.

Summary and conclusions. Differentiation

between life and death in unicellular beings should be based upon criteria which are more convenient and fundamental than measurements of capacity for growth. The principle of ion (eosin) exclusion has been substantiated as a simple, rapid tool for this purpose. The conditions for valid observations in cell and tissue cultures have been delineated in respect to: eosin, serum and electrolyte concentrations. These simplified methods have replaced cultivation procedures in studies on: the effects of exposure to pancreatin and to drying, and the exposure of sensitive cells to tuberculin; storage of stock suspensions of cells without renewal of medium; and use of such methods for investigating nutritional or metabolic requirements.

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Effects of Hormone Dose and Length of Injection on Mouse Mammary Gland Growth.* (23986)

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Use of deoxyribosenucleic acid (DNA) as index of mammary gland growth in mice has been proposed previously(1). Ultimate prac-

ticability of its employment in an objective assay technic for mammary gland growth promoting substances would be aided by elimination of any unnecessary steps in preparation of tissues and extraction of DNA, as well as determination of minimum time and estrogen required to produce an utile standard curve for comparison. We, therefore, determined total mammary gland DNA of mice injected with various levels of estrogen and proges-

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[†] Public Health Service Research Fellow of Nat. Cancer Inst.

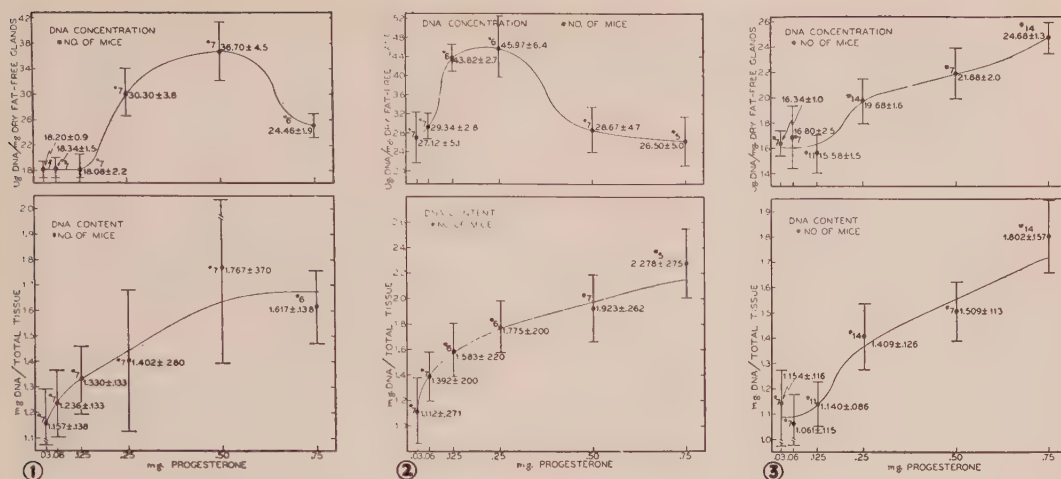


FIG. 1. Relative amounts of DNA in mouse mammary gland at various stages of lobule-alveolar growth, stimulated by inj. of various levels of progesterone plus a constant amount of estrogen (.75 µg)/day for 5 days. Range = ± stand. error.

FIG. 2. Same as Fig. 1 but for 10 days.

FIG. 3. Same as Fig. 1 but estrogen .50 µg/day for 10 days.

terone for 5 or 10 days.

Materials and methods. Mammary gland duct growth was stimulated in male albino mice weighing 16-18 g as described previously (1). In order to estimate the effects of two different levels of synergizing estrogen on promotion of lobule-alveolar growth, mice were subsequently injected daily with .50 or .75 µg estradiol benzoate (E.B.) with various amounts of progesterone (P)/day (.03, .06, .125, .5 and .75 mg). Hormones were dissolved in olive oil in concentrations such that .1 mg solution was injected daily/mouse. Mice receiving .75 mg E.B./day were divided into 2 lots, one was injected for 5-day period, the other for 10 days. Preliminary study revealed lyophilization is unnecessary provided tissues were kept in deep freeze for 4 days and then thawed in 95% ethanol. Further, cold 10% trichloroacetic acid (TCA) removed negligible amounts of nucleotides and nucleosides from small quantities of dry, fat-free pooled mammary tissues, indicating that this step could be eliminated safely. DNA was determined by Webb and Levy(2) procedure with above modifications. Mice were sacrificed on day following last injection, skinned rapidly and 3/4 of subcutaneous fascia containing mammary glands was removed and frozen. The DNA of the total tissue was determined

to indicate extent of gland hyperplasia and the DNA/mg was calculated to indicate rate of hyperplasia (speed of growth) during the growth process(1).

Results. Results of the 5-day injection period on mammary growth (Fig. 1) indicate that low doses of progesterone have very small effect on total DNA (reported in figure as DNA/total tissue), the growth "spurt" coming with larger doses. This is reflected in DNA concentration curve (DNA per mg dry, fat-free tissue) which indicates a faster rate of growth in mammary glands of mice receiving higher doses of P. The 10-day injection period (Fig. 2) using synergizing dose of .75 µg E.B. and varying doses of P indicates greater total growth (total DNA) produced by higher levels of P, while the greatest growth increments (faster rate of growth) occurs in mammary glands of mice receiving lower doses of P. This is reflected by changes in DNA concentration (growth rate) curve (Fig. 2). Mice injected with synergizing dose of .50 µg E.B. for 10 days (Fig. 3) gave total growth and growth rate curves not dissimilar to those obtained with 5-day injection period and .75 µg E.B. (Fig. 1). Total DNA remained at lower level in mammary glands of mice receiving lower amounts of P, while rapid increases are noted in sets of tissues from mice injected

TABLE I. Comparison of Log Dose *vs* Mean Response Regression Lines.

Daily dose	No. days of inj.	No. of mice	No. of means	b	S _b	Syx	Lambda
.75 μ g E.B., varying P	5	41	6	.3992	.0818	.0989	.248
.75 " " "	10	31	6	.7479	.0704	.0852	.114
.5 " " "	10	60	6	.4695	.116	.1404	.299

with higher amounts.

When means of sets of total DNA data concerning effects of time and dose were plotted against log-dose P (Table I), it was found that slope (b) of the line obtained with .75 μ g E.B. and varying doses of P for 10 days was significantly greater than obtained by injection for 5 days ($p < .0125$). It was also significantly greater than b obtained ($p < .05$) with .50 μ g E.B. plus varying P for 10 days. Lambda (λ), ratio of Syx (standard error of line around the point of means) to b and a good inverse measure of precision of a standard line for an assay procedure(3), was smaller in group receiving .75 μ g E.B. and varying P for 10 days. Therefore, the line produced by latter dose and length of injection had greatest precision. All slopes deviated with high significance from the intercept (line) at zero dose of P (the effect of the synergizing dose of E.B. remaining alone), as is evidenced by the small S_b in each case (Table I).

Discussion. An assay technic for determination of mammary gland growth promoting potency of various substances may be developed if some suitable standard curve can be established. It was demonstrated that DNA serves as a suitable index of mammary gland growth produced by injections of various levels of E.B. and P, if a constant ratio of 1:1000 of these substances is maintained. However, in practical procedure it would seem necessary to use a constant dose of estrogen synergizing with various levels of the mammary growth promoting substance to be assayed. The investigation of the effects of progesterone, a highly purified mammary gland growth promoting substance, would yield most information regarding utility of log-dose growth-response lines. Lines produced by injection for either 5 or 10 days with 0.75 μ g E.B. synergizing dose would be acceptable in an assay procedure according to this method of deter-

mination of precision (Table I) although sensitivity would be lacking in the 5-day procedure due to smaller slope. Moreover, a 10-day procedure is considerably more precise than 5-day injection.

That DNA concentration, expressed as μ g DNA/mg dry, fat-free mammary tissue, is an index of growth rate (as opposed to absolute amount of growth) has been proposed previously(1). DNA concentration curves of 5- and 10-day data with .75 μ g E.B. (Fig. 1,2) reflect rate changes in growth, thus corroborating this idea.

The synergizing dose of 0.75 μ g E.B./day has previously been reported(4,5) to be optimal in the 10-day production of mammary gland growth. Injection of a lesser amount (.50 μ g E.B./day) produced a significantly smaller slope, and a much higher lambda. DNA concentration again approximates a lagging differential of the DNA content curve and indicates that no plateauing of the growth rate had occurred in the higher dose of P.

Summary. The possible use of growth curves in assay procedures for mammary-gland growth promoting substances was investigated. Male mice were fed diethylstilbestrol for 4 weeks to insure duct growth. In addition, one group was injected subcutaneously with 0.75 μ g estradiol benzoate (E.B.)/day along with various levels of progesterone (P) for 5- or 10-day periods. Another group similarly received 0.50 μ g E.B./day plus various amounts of P for a 10-day period. Desoxyribosenucleic acid determined by a newer, shortened procedure was used as a growth index. Results indicated that best slope with greatest precision was obtained when 0.75 μ g E.B./day with various amounts of progesterone was injected for 10-day period.

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Storage of Pyrimethamine in Cells *in vitro*. (23987)

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Residual pharmacological activity of certain insoluble drugs is maintained by injection of these substances, intramuscularly, to form a "depot" from which they are slowly and continuously released. Other drugs manifest prolonged activity without the need for such means of injection, and it is known that in these cases the substances are differentially distributed in the body. The binding of pharmacologically active substances in tissues or cells is not clearly understood and there is little information on occurrence of this phenomenon *in vitro*. Pyrimethamine (Daraprim) manifests prolonged residual activity following oral administration(1). Schmidt *et al.*(2) determined the concentrations of pyrimethamine in plasma, erythrocytes, and other tissues of rats and monkeys. It was shown that all fixed tissues examined contained significantly higher concentrations of drug than blood plasma. Smith and Ihrig(3) have also demonstrated that pyrimethamine persists for considerable periods in the body of both man and monkey. We observed previously(4) that in monkey kidney tissue cultures infected with *Toxoplasma gondii*, treated with pyrimethamine for 7 days, and then incubated for 11 days without drug, cellular degeneration due to proliferation of the parasite did not occur. Nevertheless, by inoculation of mice with these cultures immediately after removal of drug, the presence of toxoplasmas could frequently be demonstrated. These results were explained by possible storage of pyrimethamine, at a level inhibitory to the parasite, within cells growing in tissue

culture. Experiments reported here were designed to demonstrate this storage of pyrimethamine in cells *in vitro* and to define the conditions under which it can occur.

Materials and methods. *Tissue culture:* Monkey kidney tissue cultures were prepared and maintained by the method of Melnick (5). Mouse mince cultures were prepared by the method of Chernin and Weller(6). Chick embryo heart explant cultures were prepared by standard plasma clot technics. HeLa cells, KB cells, conjunctival epithelium (Chang), and human intestinal cells (Henle) were purchased from Microbiological Associates, and maintained in Eagle's medium(7) with 10% calf serum, 100 units penicillin, and 100 μ g streptomycin/ml. (HeLa maintenance medium). *Drug Solutions.* Daraprim, a brand of pyrimethamine, was supplied through the courtesy of Dr. G. H. Hitchings of Burroughs & Wellcome Research Laboratories, Tuckahoe, N. Y. Stock solutions of 100 mg % pyrimethamine were prepared by bringing 100 mg of pyrimethamine dissolved in 2 ml lactic acid, U.S.P.-85%, to 100 ml with maintenance medium. This drug solution was sterilized by 02 Segas filtration and stored at 4°C. All other solutions of pyrimethamine were prepared from such stock solutions. *Mice.* NIH general purpose albino female mice of 14-20 g were used. *Protozoa.* The RH strain of *Toxoplasma gondii* was used in all tests. Methods of handling the parasite have been described elsewhere (Cook and Jacobs, *in press*).

Results. Preliminary experiments to test storage of pyrimethamine in cells growing in tissue culture were performed as follows:

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TABLE I. Duration of Storage of Pyrimethamine in Monkey Kidney Epithelial Cells after Treatment for 5 Days with Various Levels of Drug. Lysis times of cultures and survival days of mice inoculated from them following challenge* with *Toxoplasma* on designated days after removal of drug.†

Conc. of drug	Day of challenge of cultures with <i>Toxoplasma</i>													
	0		1		3		4		5		7		14	
	LT‡	M‡	LT	M	LT	M	LT	M	LT	M	LT	M	LT	M
.00	3	5.0	3	5.0	3	7.5	3	6.0	3	5.0	3	6.0	3	6.0
.05	7	6.0	6	5.0	4	6.0								
.1	N§	5.5	8	6.0	10	6.5	3	6.5	3	5.0	3	6.5	3	6.0
.15	N	7.5	N	7.5	N	7.0	N	6.5	8	5.5	7	7.0	4	6.0
.30	N	9.0					N	9.0	N	6.5	N	9.5		
.50	N	10.5									N	8	N	8

* Challenge was with 2×10^6 toxoplasmas.

† Day of removal of drug = day 0.

‡ LT = lysis time of tissue cultures; M = avg survival days of mice inoculated from cultures.

§ N = No lysis was observed in 13 days of observation of cultures.

Cultures of monkey kidney epithelium were exposed for one week to 0.5 mg % pyrimethamine before being infected with *T. gondii*. Pyrimethamine-treated cells were washed 3 times and renewed with 1.5 ml of maintenance medium. These cultures were challenged with 200,000 toxoplasmas immediately after washing. No lysis occurred within 7-day incubation period while similarly infected control cultures showed complete cellular degeneration in 4 days. In another experiment, cultures were infected with 200,000 toxoplasmas for one or 2 days prior to exposure to pyrimethamine at concentration of 0.5 mg % in nutrient medium and kept on drug for an additional day after infection, after which they were renewed with maintenance medium. These cultures showed no lysis during observation period of 17 days while control cultures lysed in 5 days.

In additional experiments, monkey kidney tissue cultures were treated with 0.5 mg % pyrimethamine for one week and then were challenged with 2,000,000 toxoplasmas. After 9 days' additional incubation, none of these cultures showed cytopathic effects and mouse inoculations with cell suspensions from these tubes were negative. Other cultures treated with 0.05 mg % pyrimethamine for 5 days before receiving the same challenge showed 4+ lysis on day 4. Control tubes of untreated *Toxoplasma*-infected cells showed 4+ lysis in 3 days and mice inoculated with cell suspensions from these tubes died in 6 days.

Further experiments to define drug storage in monkey kidney cells relative to concentra-

tion of drug in nutrient medium were performed. The results of one of these experiments are presented in Table I.

At concentrations of 0.15 mg %, pyrimethamine was apparently stored for 4 or more days in the cells. Cultures infected 4 days after removal of the drug did not lyse during 13 day observation period; cultures infected 5 and 7 days after removal of drug showed some delay in lysis time. However, concentration of stored drug was obviously not high enough to cause death of parasites, since mice inoculated from these cultures died of toxoplasmosis. At 0.30 mg %, storage was similarly evident for periods to one week, and at 0.50 mg % for as long as 2 weeks. With the latter concentration, enough drug was stored to kill the parasites. In other experiments, some storage of drug for 3 weeks, but not for 4 weeks, was seen in cultures exposed for 5 days to 0.50 mg % pyrimethamine, as evidenced by a delay in lysis of cultures following challenge with *Toxoplasma*; mice inoculated from these cultures died of toxoplasmosis, but there was an increase in their survival time.

Replicate experiments, performed with different cell lots, showed that these results were not always consistent. To determine the cause of these discrepancies, other experiments were performed to ascertain whether extent of drug storage could be correlated with age of cells.

Cultures one to 4 weeks old were treated with pyrimethamine at 0.5 mg % for 5 days and then were challenged with 2,000,000 toxoplasmas after additional intervals of one to 4 weeks. Mouse inoculations were performed to

demonstrate presence of parasites. A difference was apparent between 4-week-old cells and younger cells, in that mice inoculated with the latter survived the longest, while the oldest cells showed no difference from non-treated control tubes. Drug storage apparently was at a maximum in 14-day tissue culture cells although storage was also demonstrated at moderate levels in 7- and 21-day-old cells. These differences possibly are related to variations observed in previous storage experiments. The supernatant fluid of 7-day tissue cultures did not show the rapid drop in pH characteristic of older cultures. This may have been due in part to smaller number of cells present in cultures. Possibly there is a correlation between this difference in the ability of these cells to lower the pH and their ability to store pyrimethamine, especially if the drug is activated in host cells in some manner. Twenty-one and 28-day cells had passed their peak as regards cell division and necrotic areas could be seen in some of the cell sheaths in these cultures but rapid drops in pH of medium continued to occur. The failure to demonstrate storage or the lower rates of storage in older cultures may be due in part to presence of necrotic areas in the cell sheaths.

Additional tissue culture experiments designed to compare abilities of other cells to store pyrimethamine were performed. In every case 0.5 mg % pyrimethamine in HeLa maintenance medium and a 7-day drug exposure time were used. At end of exposure period the cultures were washed 3 times with maintenance medium, were replaced with HeLa maintenance medium and were reincubated for 2 additional days. At the end of this 2-day "rest" period the cultures received another medium change, were challenged with 200,000 RH strain toxoplasmas and were reincubated for one week. Control tubes were non-drugged cells receiving a similar inoculation of toxoplasmas. Inoculations into mice of pools of these cell cultures were performed at end of this incubation period to check for presence of viable parasites. All tissue cultures used in these experiments were incubated in stationary racks. The results of one

TABLE II. Comparison of Ability of Various Tissue Culture Cells to Store Pyrimethamine.

Tissue culture system	No. of mice dead of 5 inoculated from tissue cultures challenged with 200,000 RH toxoplasmas	
	Cells treated with .5 mg % pyrimethamine	Untreated cells
Monkey kidney epithelium	0	5
Mouse mince cultures	5 (9)*	5 (8)
Chick embryo heart explants	0	5 (6)
HeLa cells	0	5 (6)
KB cells	4 (10)	5 (7.5)
Conjunctival epithelium (Chang)	5 (10.5)	5 (10.5)
Human intestinal cells (Henle)	0	5 (11.0)

* Figures in parentheses are avg survival days of mice.

of these experiments may be seen in Table II.

It can be seen that when 0.5 mg % pyrimethamine is present in the nutrient medium, monkey kidney cells, chick embryo heart explants, HeLa cells and human intestinal cells are able to store pyrimethamine in levels inhibitory to the parasite. Slight increases in survival times of mice inoculated from treated versus non-treated cultures indicate that mouse mince cultures and KB cells are not as efficient as the other types of cells in storing pyrimethamine, while conjunctival epithelium (Chang) showed no evidence of drug storage under the conditions used. This may be due to differences in metabolic pathways and rates of metabolism in the cells, as well as to possible differences in permeability of cell membranes and/or the affinity of proto-plasmic granules, vacuoles and colloids of the various cells for the drug.

In addition to the above evidence, storage of pyrimethamine in monkey kidney cells was confirmed by microbiological assay technic using inhibition of growth of *Streptococcus faecalis* R as indicator of the presence of antifolic acid activity(3). These assay procedures indicated that pyrimethamine or antifolic acid metabolites of pyrimethamine were present in sonic extracts prepared from monkey kidney cells at least one week after ter-

mination of drug treatment with 0.5 mg % pyrimethamine. Sonic disruption of cells was accomplished by exposing the cellular suspensions to oscillations of a 9 kc Raytheon oscillator for 20 minutes at 0°C. The cell extracts were then centrifuged for 20 minutes at 2,500 rpm to remove gross flocculent material. The supernatant fluid was further cleared by high speed centrifugation at 15,000 rpm for one hour in a Spinco Model L centrifuge. Anti-folic activity was not demonstrated in cell extracts prepared by rapid freezing-and-thawing technics.

Discussion. Ability of a cell to store a drug or active products of the drug *in vitro* suggests an explanation for the prolonged activity of pyrimethamine. The drug or its active product is probably bound to the proteins in the cell and thus prevented from rapid elution from the cell. The failure of conjunctival epithelium to store pyrimethamine must be confirmed; at present there is no obvious explanation for this difference. The demonstration of anti-folic activity in sonic extracts of cells pretreated with pyrimethamine but not in extracts prepared by freezing and thawing is probably an indication of how tightly the drug is bound to the cellular proteins.

Schmidt *et al.*(2) have already shown prolonged activity of pyrimethamine against *Plasmodium cynomolgi* and localization of the drug and/or metabolic products indistinguishable therefrom in tissues of monkeys treated with pyrimethamine. The present report shows storage of pyrimethamine at a cellular

level in a tissue culture system and defines the conditions necessary to demonstrate such storage.

Summary. 1. Storage of pyrimethamine or active products of pyrimethamine occurs in tissue cultures of monkey kidney treated with the drug. This has been demonstrated by parasite challenges of treated cultures and by microbiological assay with *Streptococcus faecalis* R. 2. The limits of this storage phenomenon have been defined. 3. Some differences in storage of pyrimethamine were noted in monkey kidney cells of various ages. 4. Chick embryo heart explants, HeLa cells and human intestinal cells are able to store pyrimethamine in levels inhibitory to parasite. 5. Mouse mince cultures and KB cells possess a slight ability to store pyrimethamine. 6. Conjunctival epithelium (Chang) shows no evidence of drug storage.

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Reversal of Oxythiamin Toxicity in Neurospora. (23988)

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Oxythiamin acts as an antimetabolite of thiamin in the rat and in other organisms(1-5). De Caro *et al.*(5, also 3,4,6) investigated the original finding that oxythiamin increased urinary excretion of thiamin and level of

blood pyruvate in the rat, but did not produce the characteristic neuromuscular syndrome produced by administration of pyrithiamin. While both oxythiamin and pyrithiamin could lower the level of thiamin in various tissues, pyrithiamin was much more potent in this respect than oxythiamin. These authors con-

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cluded that pyriethiamin could produce the neuromuscular syndrome since it was a more potent inhibitor of thiamin, whereas oxythiamin could not do so in view of its weaker antagonistic action.

The present paper reports our studies on metabolic effects of oxythiamin in *Neurospora crassa*(8a) (wild strain) and the reversal of these effects by thiamin and by acetate.

Methods. The fungus was grown on the usual medium(8) reported by Horowitz and Beadle. Suitable additions were made before autoclaving, on reversal of oxythiamin toxicity (Table I) while volatile and heat-labile substances were added at room temperature after sterilization by filtering through Seitz filter pad. After growth period of 72 hours, the mycelia were filtered, dried at 105°C to constant weight and the weights recorded. Culture filtrates were analyzed for pyruvic and total ketoacids according to Friedmann and Haugen(9). In experiments on pyruvic decarboxylase activity, the mycelia were dried with acetone and the acetone powders kept refrigerated. Oxidation of pyruvic acid *via* Krebs cycle was minimized by addition of malonate and magnesium chloride. 15 mg of the acetone powder were shaken with 2 mg of pyruvic acid (as sodium salt), 2 mg of potassium malonate and 3 mg of magnesium chloride in total volume of 1.6-1.8 ml in a Warburg flask at 37°C. Carbon dioxide was estimated by the direct method, with and without potassium hydroxide in the center well of the flask.

Growth inhibitory action of oxythiamin on *Neurospora crassa* 8a is completely reversible by either thiamin or by acetate. Succinate and citrate also could reverse this inhibition to a large extent. Whether this might be due to formation of acetate from these 2 substances is hard to decide. A large number of other compounds including acetaldehyde, palmitate and acetoacetate could not reverse toxicity of oxythiamin. This incapacity of acetaldehyde, which is known to be precursor of acetate in the oxidation of pyruvate, shows, though not conclusively, that oxidative decarboxylation of pyruvate involved here might be a single step process bypassing acetaldehyde.

TABLE I. Reversal of Growth Inhibition Produced by Oxythiamin. pH was adjusted to 6.6 before autoclaving. All vessels contained 5 mγ of biotin and, except No. 1 (basal medium), 100 γ of oxythiamin.

Addition		Wt of dried mats, mg
Basal medium without oxythiamin		43.2
Oxythiamin		4.0
Thiamin	(50 γ)	39.3
Acetic acid	(10 mg)	43.0
Citric "	"	22.8
Succinic "	"	33.4
Lactic "	"	3.6
Malic "	"	3.0
Pyruvic "	"	2.9
Oxalic "	"	3.4
Formic "	"	4.1
Propionic "	"	3.0
Ethyl acetoacetate Na.	"	3.0
Formaldehyde	"	3.0
Acetaldehyde	"	3.0
Methanol	"	3.0
Ethanol	"	3.6
Palmitate	"	4.0
NaHCO ₃	"	3.6
(NH ₄) ₂ HPO ₄		4.0

However, formation of acetaldehyde by *Neurospora* has been shown by Strauss(7). More recent work(11) shows that the metabolic function of lipoic acid includes oxidative decarboxylation of pyruvate. However, while bicarbonate could replace the pyruvate oxidation factor in *Streptococcus faecalis*(10), it could not reverse oxythiamin toxicity in our experiments. Further, while acetate helps growth of *Neurospora* in the presence of oxythiamin, it does not help accumulation of keto and pyruvic acids. This may be attributed to inhibition of decarboxylation of pyruvate by oxythiamin. The larger amounts of acetate added seem to prevent further conversion of pyruvate to acetate, thus increasing the accumulation of pyruvic acid. This is plausible on grounds of mass action law. Citrate and succinate, which do not help growth of oxythiamin-inhibited fungus as much, do not lead to as high accumulation of ketoacids. Neither do they, on the other hand, decrease their content.

Inability of palmitate to reverse toxicity of oxythiamin, by giving rise to acetate, seems to support Lein's conclusion(12) that palmitate and stearate are inert in *Neurospora* in relation to formation of acetate. Strauss(7)

TABLE II. Metabolic Effects of Oxythiamin, and Their Reversal. 10 ml medium, 72 hr growth, pH 6.6.

Addition to basal medium	Mat wt, mg	Total keto-acids, mg	Pyruvic acid, mg
Oxythiamin (100 γ)	3.0	5.80	5.50
Oxythiamin + thiamin (50 γ)	33.6	4.0	.8
Oxythiamin + acetic acid (10 mg)	37.9	26.8	21.2
Oxythiamin + citric " (10 mg)	27.4	4.25	.85
Oxythiamin + succinic " (10 mg)	28.1	6.55	6.55

indicates 2 routes for formation of acetate in oxidative decarboxylation of pyruvate, one mediated by pyruvic decarboxylase, and the other not. Strauss(7) has also shown low carboxylase activity in some strains of *Neurospora crassa*.

Accumulation of ketoacids, and the higher percentage of pyruvic acid in total ketoacids in media with oxythiamin is interesting. These results (Table II) denote a similarity of function of thiamin in several organisms, including the rice moth larva (*Corcyra cephalonica* St.(13) and the rat(1). Table III shows the effect of incorporation of oxythiamin, and of both thiamin and oxythiamin, in culture me-

TABLE III. *In Vitro* and *In Vivo* Effect of Oxythiamin on Pyruvic Decarboxylase Activity of *N. crassa* Mycelial Acetone Powders.

No.	Detail	μ l CO ₂ evolved per mg tissue per hr
1.	<i>N. crassa</i> grown in medium containing 100 γ of oxythiamin/10 ml	7.81
2.	<i>Idem</i> and 50 γ thiamin/10 ml	15.5
3.	Acetone powder from No. 1 with 50 γ thiamin added <i>in vitro</i>	12.3
4.	Mycelium grown in basal medium	9.64
5.	Acetone powder from No. 4 with 100 γ oxythiamin added <i>in vitro</i>	.0
6.	<i>Idem</i> + 50 γ thiamin	11.27

dia, on decarboxylation of pyruvate by mycelial preparations. Addition of oxythiamin to basal medium might be considered as equivalent to *in vivo* deficiency of thiamin, while addition of antivitamin to a mycelial preparation obtained from normal medium might be viewed as *in vitro* deficiency. While growth was depressed to a large extent by *in vivo* addition of oxythiamin, pyruvic decarboxylase activity of the small amount of mycelium obtained, was not strikingly decreased. On the other hand, *in vitro* addition of antivitamin abolished enzymatic activity. This inhibition was completely counteracted by thiamin and not by acetate (the results with acetate are not shown in the present communication, since they are negative).

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Serum Glutamic Oxaloacetic Transaminase Activity after Whole Body Irradiation. (23989)

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As part of a continuing search for biologic phenomena which might give a clue to the amount of total body irradiation absorbed by mammals, the effect of such irradiation upon serum glutamic oxaloacetic transaminase (SGOT) has been examined in 2 different laboratories. SGOT activity is increased in acute myocardial necrosis(1,2,3) and in acute hepatocellular damage(4,5); it was therefore felt that this enzyme system might be a satisfactory index for determining extent of irradiation injury. The following report details the results obtained.

Exp. 1. Method. New Zealand male rabbits, 4½ to 5 lb. in weight were used. Each rabbit was placed in a wooden container, just large enough to accommodate the animal. X-ray was administered toward the lateral aspect of the body, half of the total dose being given from the right, and the other half from the left. Dosages were measured in air at surface of the body. The dose in the midline of the animal was roughly 75% of the air dose. X-ray factors were 200 Kv., 25 Ma., H.V.L. 1.1 mm Cu. Blood specimens were obtained from the heart by direct cardiac puncture.

Results. Table I shows the SGOT levels in the different animals pre- and post-irradiation. The control animal received a sham treatment. With a dose of 500 r, determinations between 6 and 18 hours showed a decrease in the SGOT levels. This phenomenon also was generally seen in those animals given 1000 r.

In 2 instances in animals given 1000 r, no appreciable change was noted. One of these rabbits died after a few hours, but the other was followed for 168 hours, and the transaminase level appeared to be quite constant. In this experiment the results appear to be different than those of the subsequent experiment and with those of Milch and Albaum(6). The method of irradiation in this first experiment was directed from the lateral aspects of the body rather than from above and perhaps this may have given a higher dosage to some organ which forms the enzyme and thus might have inhibited enzyme formation.

Another factor was the use of cardiac puncture for blood removal. This was a possible source or error, since one of us (G.K.) found that when specimens were collected by cardiac puncture some of the control animals died. Post-mortem examination revealed the pericardial sac to be filled with clotted blood and there was visible scarring of the myocardium. At times when collecting blood in control animals by intracardiac puncture elevated SGOT levels were obtained even though no other noxious stimuli were applied, indicating a definite reaction on this enzyme system as a result of cardiac puncture. For this reason in the subsequent experiment blood specimens were obtained by the free bleeding technic.

Exp. 2. Method. Male, New Zealand strain rabbits weighing from 5½ to 7 lb were used as the experimental animals. The x-irradiation

TABLE I. SGOT Levels in 9 Rabbits after Various Doses of Whole Body Irradiation (Units/ml).

Dose (r)	Pre	Hr after irradiation									
		1	2	3	6	18	24	48	72	128	168
Control	29			29	35		29				
200	29				46		39	39	32		
500	37					22		13	20		
500	43			17	33		43	29	30		
1000	69	25	35	32			17	17	12		
1000	30	18		Died							
1000	46	26					27	20	Died		
1000	14	12					9	10	9		12
1000	17	17			Died						

TABLE II. SGOT Levels in 28 Rabbits after Various Doses of Whole Body Irradiation (Units/ml).

Dose (r)	Pre	Hr after irradiation						Survival time†
		1	5	11	25	49	73	
1000	25	22	26	28*	12	11		1 wk
	16	27	33	18	20	36	21	1 "
	21	21	35	18	13	19	14	1 "
	14			34	19	21	17	S
	25	25						5 hr
		24	42	70*	137	57	49	S
	15	15	20	20*	32	15		S
	19	20	18	31	19	11	14	S
	29	32	25	30	15	12	17	S
1250	23	20	33	305	500	52	24	S
	33	48	126	2500				24 hr
	19	23	23	86				24 "
	14	23	117	117	75	37		60 "
	16	23	17		22	11	15	S
	15	26	19		12	10	15	S
	22	18	27	68	36	16	68	S
	11	19	21	39				24 hr
	33	30	29	28	32			S
	27	33	19	31	33			S
1500	20	23	37	41		24		S
	9	14	71	534				24 hr
	13	18	71					11 "
	26	21						5 "
	11	27						5 "
	29	28	24	27	24	25	20	S
	47	42	57	134	43	21	27	S
	16	22		38	35			48 hr
	14	15	36	36	48	19	24	S

* 10 hr post-irradiation values.

† S = Survived for 2 wk experimental period.

procedure consisted of placing the animal in a 20x20x20 cm corrugated box. Dose levels of 1000, 1250, and 1500 r in air were administered to the animals. X-ray factors consisted of 200 Kv., 20 Ma., 20x20 cm field, $\frac{1}{4}$ mm Cu 1 mm Al filter, 50 cm TSD. Irradiation was given from above, one portal being used. The total x-irradiation times for 1000, 1250 and 1500 r (air dose) were 9'32", 11'55", and 14'18" minutes respectively. It should be noted that the actual dose in the midzone of the animal, correcting for the distance according to the inverse square law and neglecting tissue absorption, was approximately 600, 750 and 900 r. All experimental animals served as their own controls by having an initial specimen taken before irradiation. It had been previously determined that irradiation and blood specimens collected serially at specified time intervals did not reveal elevated serum glutamic oxaloacetic transaminase levels. Blood specimens were obtained by a free bleeding technic, *i.e.* cutting the ear vein of

the rabbit and collecting approximately 1.5 ml of blood. GOT was determined on sera after centrifugation of the clotted specimens (7).

Results. Average pre-irradiation value for all experimental groups was 21 GOT units (Table II). At one hour post-irradiation there was no perceptible rise in SGOT. For time intervals of 4 to 25 hours post-irradiation, average GOT units were higher than pre- or one hour post-irradiation values. The highest GOT levels occurred 11 hours after total body irradiation. The group of animals receiving 1000 r (air dose) showed the least GOT increase.

The individual SGOT levels of all groups, for the 4 to 25 hour post-irradiation intervals, did not show a consistent increase. Individual values for all groups ranged from that of the pre-irradiation value to an increase of several hundred GOT units. As there was an inconsistent increase in GOT it was decided that statistical groups were not applicable.

Average values of all groups for 25 hours after total body irradiation were still elevated but considerably lower than those for 11 hours post-irradiation. At 49 hours post-irradiation the average values for all groups were the same as that observed for pre- and one hour post-irradiation. After 73 hours average GOT values were similar to the pre-irradiation level.

While the above work was in progress, Milch and Albaum published studies of SGOT levels in x-irradiated animals, using dose levels of 500, 750 and 1000 r. Their data demonstrate significant increase in serum glutamic oxaloacetic transaminase activity within 6 hours after receiving 500 r and within 3 hours after receiving 750 and 1000 r. An exact comparison is not possible, because of differences in time intervals used, but our data indicate an average increase 5 hour post-irradiation. Some animals in all groups did not show any response.

All animals that survived the total body x-irradiation were kept for a maximum of 2 weeks post-irradiation. There was no correlation between GOT changes and survival time, (Table II). The data indicate that some animals survived after an increase of several hundred SGOT units while others died showing only mild GOT increase. More animals in the groups which received dosages of 1250 and 1500 r died during the first 24 hours. Although increased SGOT activities may qualitatively indicate tissue damage inflicted by irradiation, survival of the animal appears to depend on ability to overcome and repair the

resultant damage.

Summary. New Zealand strain rabbits were given varying dosages of total body x-irradiation. SGOT determinations were made at various intervals in the post-irradiation period up to 168 hours following irradiation. Initially the blood specimens were drawn by direct cardiac puncture and the results in these instances were too variable to be used for diagnostic or prognostic purposes. When the free bleeding technic was used for obtaining blood specimens, more consistent results were obtained. Average SGOT values for pre- and one hour post-irradiation were of the same order of magnitude. An increase in GOT activity was observed as early as 5 hours after total body irradiation and remained elevated for 25 hours. After 49 hours post-irradiation SGOT levels returned to normal. There was no correlation between SGOT activity and total irradiation or survival time of the animal.

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Effect of Histone on Plasma Unesterified Fatty Acids.* (23990)

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Recognition of the physiological importance of unesterified fatty acids (UFA) followed the observation of the UFA-increasing effect of heparin(1,2,3), and reports establishing UFA as available metabolites(4,5,6,7). Pro-

tamine was found to counteract the effects of heparin(7,8,9). In the present studies the effects of histone on UFA metabolism were investigated. Histone, like protamine, is a positively charged molecule, but unlike protamine it is widely distributed physiologically.

Methods. Alimentary lipemia was pro-

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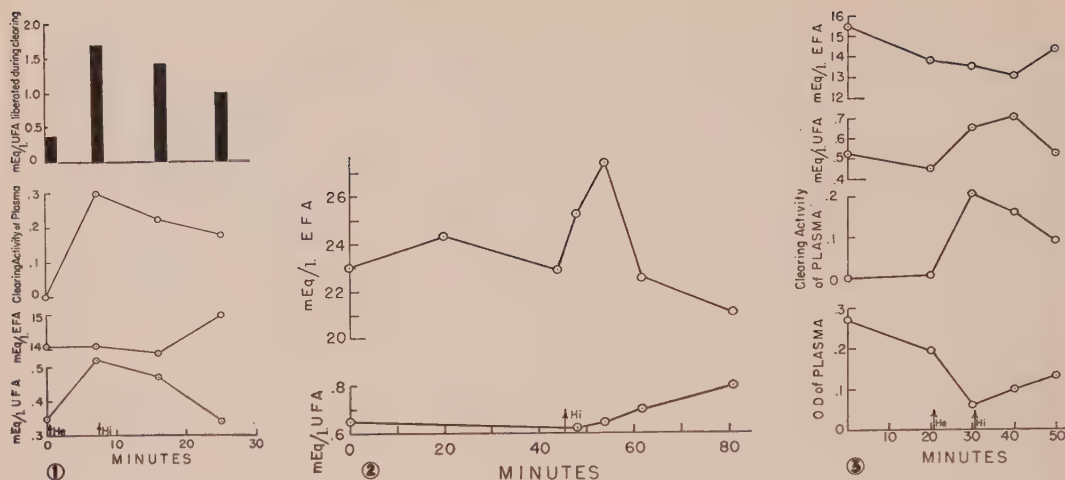


FIG. 1. Effects of 100 mg histone (Hi) following 6 mg heparin (He) in a nonlipemic dog.

FIG. 2. Effects of 90 mg histone (Hi) in a lipemic dog.

FIG. 3. Effects of 50 mg histone (Hi) following 6 mg heparin (He) in a lipemic dog.

duced in mongrel dogs by feeding about 40 ml of cod liver oil. The oxalated blood samples were promptly refrigerated and centrifuged in the cold. UFA were determined by the method of Grossman(10). Esterified fatty acids (EFA) were estimated by the procedure of Stern and Shapiro(11). All analyses were performed in duplicate. Histone, from calf thymus nuclei (Mann Research Laboratories), was injected intravenously in 2.5 to 5.0 mg/kg doses, either with or without previous intravenous injection of heparin (about 0.3 mg/kg). Animals that did not receive heparin showed an untoward reaction to histone that lasted about one minute. Clearing activity of the plasma was determined by clearing in optical density units of 1 ml of cottonseed-oil emulsion by 0.5 ml of plasma after 2 hours' incubation at room temperature.

Results. Administration of histone to nonlipemic dogs did not influence either UFA or EFA levels of plasma (4 exp.). When histone was administered following injection of heparin into nonlipemic dogs, the heparin-induced changes, such as clearing activity of plasma and elevation of UFA, were counteracted by histone (3 exp.), (Fig. 1).

In 8 experiments on lipemic dogs, histone alone increased visible lipemia, and, in most cases, blood EFA, but it had no consistent effect on UFA (Fig. 2). When heparin pre-

ceded administration of histone in lipemic dogs, the heparin-induced changes were again counteracted by histone; visible lipemia and EFA were increased, clearing activity of plasma and UFA were decreased (Fig. 3) in 6 experiments. In control experiments on both normal and lipemic animals the effect of heparin lasts over 30 minutes (Figs. 2-6 and 9-11 of ref. 7, and Figs. 1 and 3 of ref. 9).

Discussion. UFA is liberated by clearing factor(1,2,3) or by tissue lipases(12,13,14,15) and is removed by tissues(4,5,7). Both clearing factor(8,9) and tissue lipoprotein lipase(12,13) are inactivated by protamine. Histones, which unlike protamines, are physiologically widely distributed, have an action similar to that of protamine on lipid transport. The latter finding makes it very tempting to speculate that UFA-liberation may be under the influence of an intracellular lipase-histone equilibrium.

Summary. Histone influenced lipid transport in the same manner as protamine. In nonlipemic dogs, it failed to influence unesterified or esterified fatty acids, reversed the action of heparin on clearing activity, and opposed the elevation of unesterified fatty acids. In lipemic dogs, histone increased lipemia without affecting the unesterified fatty acids. In similar animals after heparin, histone reversed the action of heparin, increasing li-

pemia and decreasing clearing activity of the plasma and unesterified fatty acids.

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Effect of Zinc upon Growth and Incidence of Perosis in Turkey Poults.* (23991)

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Todd *et al.*(1) and Stirn *et al.*(2) reported that zinc is indispensable in the nutrition of the rat. Follis *et al.*(3) studied the histology of zinc deficient rats and noted a parakeratosis as a characteristic symptom. Tucker and Salmon(4) reported that a parakeratosis syndrome was noted in swine on rations containing 34 to 44 ppm of zinc. The incidence was increased by increased levels of calcium and/or phosphorus. The syndrome was prevented and growth improved by supplementing the ration with zinc. Zinc was also found to be effective in preventing the parakeratosis at lower levels of calcium in practical rations by Luecke, *et al.*(5) and purified protein (Drackett) diets by Conrad and Beeson(6). Morrison *et al.*(7) obtained no growth response to zinc with chicks fed a purified ration containing an isolated soybean protein (Drackett C-1). Mehring *et al.*(8) also

failed to observe any increased growth by addition of zinc to a practical type ration for chicks. O'Dell and Savage(9) observed increased growth of chicks fed a Drackett protein basal ration with relatively high levels of calcium and phosphorus when zinc carbonate was added. Norris *et al.*(10) also obtained a slight growth response in chicks with a similar type ration, except that lower calcium and phosphorus levels were used and concluded that the requirement of chicks for zinc was in the order of 20 to 25 ppm. Hunt *et al.*(11) and Butters and Scott(12) noted an enlarged hock condition in turkey poults fed purified rations with isolated soybean protein (Drackett 220 or C-1) as source of protein. It could be prevented by substituting soybean oil meal for Drackett protein or by adding soybean oil meal ash to purified rations(12). Slinger *et al.*(13) noted no improvement in growth of poults by addition of zinc to a practical ration. Supplee *et al.*(14) observed that zinc and potassium additions to a purified ration containing Drackett protein were effective in reducing the incidence of the enlarged hock disorder and in improving growth. The pres-

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TABLE I. Effect of Zinc, Potassium, and Soybean Oil Meal on Growth and Perosis in Poults.

Supplement	Level of added zinc, ppm	Avg. gain in g and (perosis score)			
		Exp. 1	Exp. 2	Exp. 3	Exp. 4
None	0	(3.0)323; (3.5)277	(3.4)269	(2.4)189	(2.6)258
ZnCl ₂	4.8			(2.2)230	
	9.6			(2.2)230	
	19.2			(1.7)271	
	38.4			(1.5)279	
	48.0	(1.4)423	(.8)366		
	76.8			(1.0)269	
	96.0		(.6)408		(.4)297
	153.6			(1.1)252	
ZnCl ₂ + 0.3% KCl	48	(1.4)382; (.9)366	(1.1)379		
ZnO	38			(1.4)291	
Soybean oil meal (60%)		(1.4)425			
Duration of trial, days		22	21	18	17

ent work was carried out to study the effect of zinc and potassium on poults fed a purified ration containing Drackett protein. Zinc was found to be very effective in promoting growth and reducing the incidence of perosis.

Methods. The basal ration contained the following: isolated soybean protein (Drackett C-1), 30 g; cellulose (Solka-Floc), 5 g; soybean oil, 3.5 g; dicalcium phosphate (CaHPO₄), 3 g; mineral mixture, 2.563 g; calcium carbonate, 2.5 g; vitamin mixture(15), 2 g; choline chloride (25%), 1 g; DL-methionine, 0.45 g; Vit. E concentrate (20,000 IU/#), 0.2 g; Vit. D₃ concentrate (1,500 ICU/g), 0.1 g; inositol, 0.1 g; Vit. A concentrate (20,000 IU/g), 0.05 g; aureomycin, 0.01 g; niacin, 2 mg; folic acid, 0.5 mg; biotin, 0.04 mg; Vit. B₁₂, 1 µg; and pearl corn starch to equal 100 g. The mineral mixture supplied the following/100 g of diet: NaCl, 1 g; MnSO₄ 1H₂O, 0.03 g; Na₂SiO₃9H₂O, 0.115 g; FeSO₄ 7H₂O, 0.07 g; CuSO₄5H₂O, 0.008 g; Co(AC₂) 4H₂O, 0.002 g; KI, 0.001 g; Al₂(SO₄)₃18H₂O 0.025 g; MgSO₄7H₂O, 0.63 g; KCl, 0.3 g; K₂HPO₄, 0.381 g; and (NH₄)₆Mo₇O₂₄4H₂O, 0.001 g. The basal ration was analyzed by the method of Rush and Yoe(16) and contained 26 ppm of zinc. It contained 1.88% calcium and 0.75% phosphorus by calculation from the minerals. Broad Breasted Bronze poults were fed the basal ration 4 days before being segregated into groups of 10 birds each to be fed the experimental rations. They were housed in electrically heated bat-

teries with raised wire floors. Feed and water were supplied *ad libitum*. Trace amounts of zinc as furnished by galvanized feeders and watering troughs were not taken into consideration. The birds were weighed twice weekly and the trials were continued 17 to 22 days (Table I).

Results. Poults fed the basal ration developed a leg abnormality, characterized in its most severe state by a swelling and flattening of the hock joint and slipping of the Achilles tendon from the condyle (Fig. 1). A shortening and bending of the tarsometatarsus and a bending of the tibiotarsus have been observed. The condition is similar to perosis, and in the absence of histological examination to characterize it further, we will refer to it as perosis. In contrast to Butters and Scott(12), no reduction in bone ash has been observed in our studies. The incidence and degree of perosis were markedly reduced as zinc was added to the ration (Table I). Growth was also greatly improved by addition of zinc to the ration, and in Exp. 3 about 38 ppm of zinc in addition to the amount already present in the ration were required to give optimum growth. Statistically significant growth increases were noted in both males and females. Zinc chloride and zinc oxide appear equally effective in promoting growth and preventing perosis. Potassium chloride in addition to zinc was not more effective than zinc alone. Soybean oil meal, replacing Drackett protein, was effective in increasing growth and decreased perosis

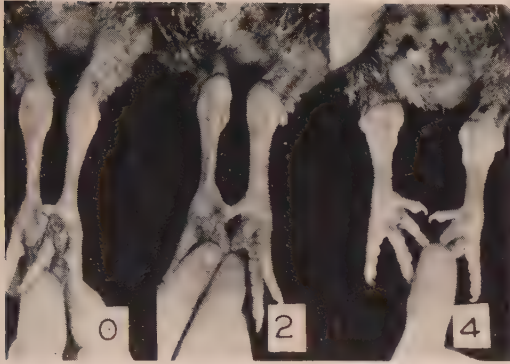


FIG. 1. Legs of turkey poults illustrating normal (0), intermediate (2) and severe (4) perosis caused by feeding ration deficient in zinc for 21 days.

about as much as zinc. The meal was analyzed and contained about 66 ppm of zinc.

Discussion. The observation of perosis or leg weakness in turkey poults fed a ration containing Drackett C-1 protein is similar to that reported by Hunt *et al.*(11) and Butters and Scott(12). The abnormality was also prevented by replacing the Drackett protein with soybean oil meal as reported by these workers. Supplee *et al.*(14) noted the same hock condition and were able to prevent it with potassium and zinc supplements. Our experiments have shown that zinc is the element which is effective in prevention of perosis and promotion of growth. The levels of calcium and phosphorus in the ration no doubt influence the response obtained to zinc.

The response to soybean oil meal could be explained on the basis of the zinc content (66 ppm *vs.* 24 ppm in Drackett protein). However, it is not certain whether association of hock disorder with the use of Drackett protein is due to low zinc content entirely or whether there are other factors involved which may influence its availability or utilization.

Turkey poults appear to resemble rats(1,2, 3) and swine(4,5) in having a definite requirement for zinc. The mechanism by which

zinc may function in the metabolism of the poult has not been determined.

Summary. Turkey poults fed a purified ration containing an isolated soybean protein (Drackett C-1) developed perosis. Addition of zinc to the ration improved growth and markedly reduced the incidence and severity of the perotic condition. Approximately 40 ppm of zinc, in addition to the 26 ppm contained in the basal ration, were required for optimum growth.

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Relationship between Salt Tolerance and Resistance to Chloramphenicol in *Staphylococcus pyogenes*.^{*} (23992)

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It has been recognized for some time that Gram positive cocci are capable of growth in comparatively high concentrations of salt. Hill and White(5) recommended media containing from 2 to 20% NaCl for selective isolation of staphylococci, and Koch(6) suggested that agar containing 7.5% NaCl or broth containing 15% NaCl be used as a selective medium for isolation of all staphylococci. Proteose-lactose agar containing 7.5% NaCl was found by Chapman(2) to be the medium of choice for isolation of micrococci from fecal material. McVeigh and Hobdy(7) found that 2 penicillin-resistant strains of *Micrococcus pyogenes* had lost the ability to grow in proteose-lactose medium containing 7.5% NaCl; strains resistant to streptomycin, aureomycin, chloramphenicol and subtilin retained their ability to grow in this medium. This paper reports a comparative study of salt tolerance in chloramphenicol sensitive and resistant strains of *Staphylococcus pyogenes*. Also, the combined effect of salt and chloramphenicol upon growth has been examined.

Methods. The organisms used were a strain of *S. pyogenes* which was sensitive to chloramphenicol (Strain S) and a resistant mutant (Strain R) derived therefrom(10). The resistance of Strain R was approximately 100 fold that of Strain S(10). The basal medium contained Difco Casamino acids, salts, vitamins and glucose(10). Glucose was autoclaved separately and added aseptically to sterile cooled medium to eliminate any error due to caramelization and preclude spurious results from uncontrollable stimulatory products of heat degradation of glucose(9). Riboflavin was omitted from the medium because

of its toxicity for the test organisms(11). Inoculum cells were harvested by centrifugation from 20-24 hour cultures in AC broth (Difco), washed twice in basal salt solution, resuspended and diluted to the desired concentration. All tubes of test series were inoculated with approximately 250 cells. After incubation at 37°C, growth was measured turbidimetrically with Bausch and Lomb Spectronic 20 photoelectric colorimeter with setting of 525 mμ. Every experiment was performed in duplicate and repeated at least once.

Results. When the 2 strains were inoculated into the basal medium containing increasing concentrations of NaCl, KCl, and glucose, it was found that NaCl and KCl were more inhibitory toward Strain R than toward Strain S (Fig. 1). In some instances, this difference was quite marked. For example, 0.8 M KCl was completely inhibitory to Strain R, whereas 1.8 M was required to inhibit Strain S. Other experiments indicated that NH₄Cl was slightly less inhibitory on a molar basis than KCl or NaCl, whereas MgCl₂ was more inhibitory. However, precipitation of the latter upon autoclaving made growth measurements by nephelometry impossible. By subculture it was determined that 0.6 N MgCl₂ demonstrated primary toxicity toward Strain R, while 1.0 N was required to render Strain S non-viable.

The difference in tolerance to glucose is insignificant; however, it should be noted that the osmotic pressure of glucose at highest concentration tested is only about one-half that of the salts.

Effect of increased salt tolerance upon resistance to chloramphenicol. Since Strain R exhibited a decreased tolerance to salts, it was of interest to determine whether an inverse relationship existed between resistance to salt and to chloramphenicol. The salt tolerance of Strain R was increased by serial transfer in the basal medium containing increasing con-

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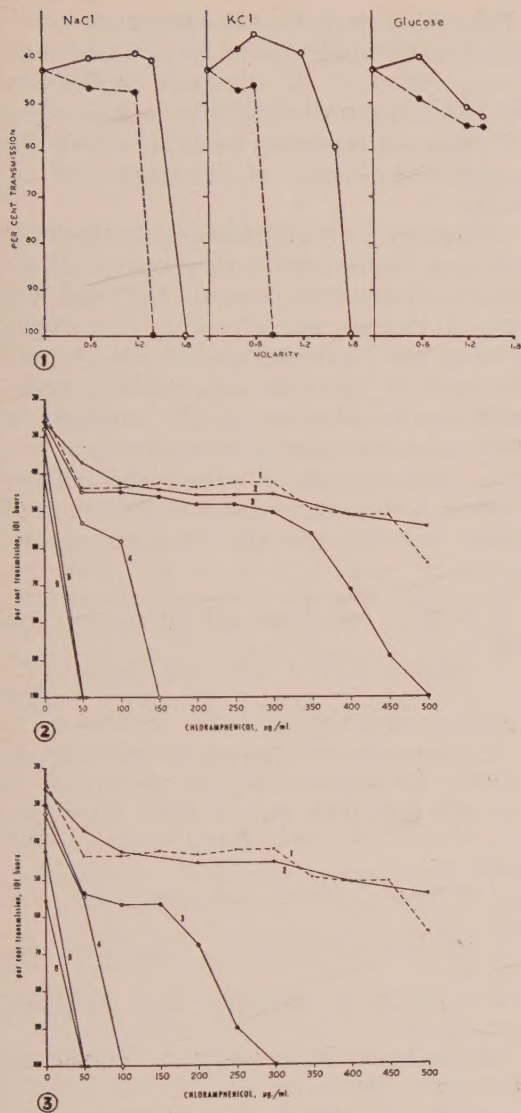


FIG. 1. Tolerance to NaCl, KCl, and glucose of *Staphylococcus pyogenes*, Strain S (solid lines) and Strain R (broken lines).

FIG. 2. Response of Strain R to chloramphenicol before and after enhancement of tolerance to NaCl. 1. Strain R after 9 transfers in the basal medium (control). 2. Strain R (stock strain). 3. Strain R after 9 transfers in increasing concentrations of NaCl. 4, 5, and 6. Same strain in basal medium with 0.8 M, 1.5 M, and 2.0 M added NaCl, respectively.

FIG. 3. Response of Strain R to chloramphenicol before and after enhancement of tolerance to KCl. 1. Strain R after 9 transfers in basal medium (control). 2. Strain R (stock strain). 3. Strain R after 9 transfers in increasing concentrations of KCl. 4, 5, and 6. Same strain in basal medium with 0.8 M, 1.5 M and 2.0 M added KCl, respectively.

concentrations of either KCl or NaCl. The strains resulting after 9 transfers could grow in the basal medium containing 2.2 M KCl or 2.0 M NaCl respectively. This represented the maximum adaptation which could be obtained. Increased tolerance to one of these salts conferred increased tolerance to the other.

Fig. 2 and 3 show that increased salt tolerance resulted in a marked decrease in resistance to chloramphenicol. Strain R was only slightly inhibited by 450 μg of chloramphenicol/ml, but after passage through NaCl was markedly inhibited at this concentration. Similarly, the KCl-tolerant strain was completely inhibited by 300 μg /ml. Control cultures, transferred 9 times in the absence of salts, demonstrated no significant alteration in chloramphenicol resistance. Since Strain R has been shown to retain resistance to chloramphenicol after 25 transfers in drug-free medium (10), it would appear that transfer in presence of salt results in selection of a strain with increased salt tolerance and a parallel decreased resistance to chloramphenicol.

Fig. 2 and 3 also indicate a striking combined effect of chloramphenicol and salts upon salt-tolerant strains (lines 4, 5 and 6). The NaCl-tolerant variant of Strain R was completely inhibited by 0.8 M NaCl in combination with 150 μg chloramphenicol/ml (Fig. 2, line 4). These concentrations were without effect when used alone. A similar effect was noted with the KCl-tolerant variant (Fig. 3, line 4) and comparable results have also been obtained with the original Strains S and R. In most concentrations the effect of both KCl and NaCl appeared to be additive, and often synergistic, with chloramphenicol; KCl was the more effective of the two salts in this respect.

Discussion. The inverse relationship between salt tolerance and chloramphenicol resistance could be explained by at least 2 possible mechanisms. First, it might be anticipated that chloramphenicol interferes with some enzymatic sequence which is relatively resistant to salt. Alteration of this enzyme sequence as a result of chloramphenicol resis-

tance could be accompanied by a decreased resistance to salt. Alternatively, the inverse resistance patterns could be due to altered permeability. If salt and chloramphenicol gain access to the cell by different means, an increased activity of one mechanism might well be accompanied by a decreased activity of the other.

The mechanism of toxicity of salts for bacteria is poorly understood. While osmotic pressure may play a role, additional toxic characteristics must be ascribed to salts. Spiegelberg(13) noted that *Clostridium pasteurianum* was inhibited by a NaCl solution of only 18 to 38 atmospheres, whereas a sugar solution of 60 to 70 atmospheres was required for inhibition.

The additive, and often synergistic effect, noted with combinations of salts (KCl and NaCl) and chloramphenicol would indicate that their modes of action are not the same. No reports of synergism between salts and antibiotics have been found, although there are several reports of the converse. For example, Weinberg(14) noted that inhibition of *Pseudomonas aeruginosa* by oxytetracycline was strongly reversed by several multivalent cations. Berti(1) found that Ca^{++} and Mg^{++} antagonized the effect of streptomycin on *Escherichia coli* and Soncin(12) reported a similar antagonism between Mg^{++} and aureomycin, terramycin and chloramphenicol. Grossowicz, *et al.*(3) observed that Na^+ and K^+ antagonized the inhibition of *S. aureus* by spermine and spermidine. The action of tetracycline on *S. aureus* and *S. albus* was reversed by Mn^{++} (4).

It should be noted that even at salt concentrations which are slightly stimulatory, the addition of chloramphenicol results in more inhibition than the drug could produce alone. According to Winslow and Haywood(15), stimulatory concentrations of salts are associated with increased permeability of cells.

This could explain the enhancement of inhibition by chloramphenicol at low salt concentrations. However, the decreased permeability at high concentrations of salts, postulated by Winslow and Haywood, would not explain the greater enhancement of chloramphenicol activity.

Summary. 1) A chloramphenicol-resistant strain of *Staphylococcus pyogenes* (Strain R) has been found less tolerant to NaCl and KCl than its chloramphenicol-sensitive counterpart (Strain S). The restoration of salt-tolerance in Strain R by serial sub-cultivation in increasing concentrations of salts resulted in a decrease in resistance to chloramphenicol. 2) The combined effects of chloramphenicol and salts on both Strains S and R were additive in most concentrations and frequently synergistic.

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Comparison of Circumferimetry with Observation in Quantitation of Serotonin-Induced Edema in Rat Paw. (23993)

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Selitto and Randall(6) described a method for evaluating anti-inflammatory drugs by their ability to suppress edema resulting from injection of yeast under the plantar aponeurosis of the rat hind foot. This swelling reached its peak in 2 to 4 hours, and lasted to 48 hours. Inhibitory action of the drug was measured by circumferimeter. Later Benditt and Rowley(1) showed that serotonin in minute dosage was capable of producing edema which reached its peak in 2 hours, with total duration about 4 hours. Kelemen (4) demonstrated ability of salicylates to protect against serotonin induced edema. The magnitude of this action was estimated visually.

The present communication reports results of study designed to compare accuracy of circumferimetric measurement with that of visual scoring in the rat paw made edematous by serotonin injection.

Methods. Albino rats ranging from 150-175 g were distributed by random assortment into 4 groups of 10 rats each. A hydrating dose of 25 cc/kg of water was fed to each rat to insure fairly consistent hydration. One hour later, aminopyrine was injected subcutaneously at logarithmically spaced doses of 50, 100, and 200 mg/kg. These treatments were assigned at random. This injection was followed immediately by plantar injection of serotonin (2.5 μ g in 0.1 cc) in both hind feet. One group served as a control. The hind feet of each rat were measured by a circumferimeter, made by affixing a loop of silk suture to barrel of a 10 ml Luer-Lok® syringe. A line was marked on the foot prior to measurement that all readings be taken at the same site. Measurements were taken initially and 2 hours after drug administration. Logs of increments in diameter were averaged. After each measurement the edema of each animal was scored by 3 scorers using a 0-4 scale. The scorers had no knowledge of the treatment of

the animals. Each cage of rats could contain animals treated with any dose level of aminopyrine and controls, assorted by random assignment of drug and dose. Since no statistically significant difference was observed among the scorers, the 3 scores on each animal were combined into a single average.

Results. Table I presents Y_1 , the mean scores obtained by 3 scorers observing 4 groups of animals 2 hours after treatment with aminopyrine, and Y_2 , the circumferimetric data obtained on same animals. The circumferimetric data are expressed in terms of the response metameter,

$$Y_2 = 100 \log \frac{2 \text{ hr post treatment reading}}{\text{Zero hr control reading}}$$

The metameter Y_2 was adopted because it adequately allows for inequalities in zero hour readings and its variability is independent of its mean value as required in a bioassay (Finney, 3).

Fig. 1 shows dose response curves obtained with these 2 types of response.

To reach a decision concerning which of the 2 available technics was better suited for quantitative work, the index of precision (Ott, (5)); Bliss and Marks(2) was computed for each procedure. As discussed in the cited references, λ is a dimensionless quantity which specifically characterizes the inherent precision of the assay in question. The smaller the value of λ the greater the inherent precision of the assay. The values obtained were as follows:

For scoring technic $\lambda = .952$

For circumferimetric assay $\lambda = .568$

Thus, the inherent precision of the assay using the scoring procedure may be expected to be much less than that of an assay based on circumferimetric assay expressed as metameter defined in above equation. To assay the potency of a sample with an error of $\pm 25\%$ would require a total of 308 animals using the

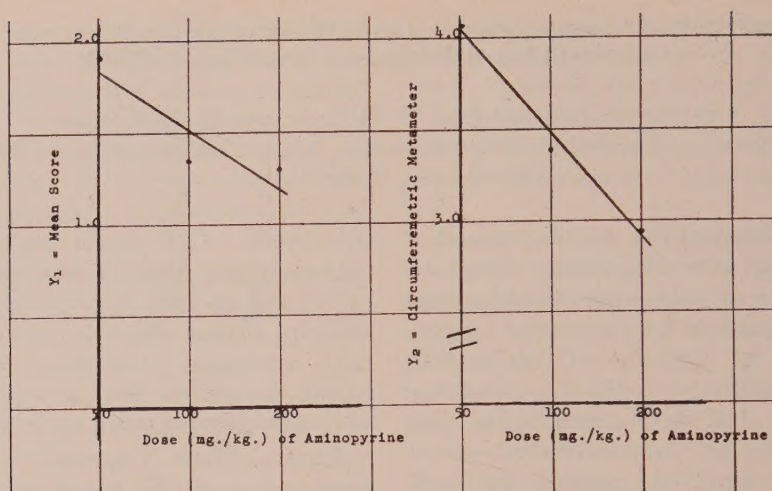


FIG. 1a (left). Dose response curve obtained using scoring technic.

FIG. 1b (right). Dose response curve obtained using circumferimetric data.

scoring procedure and 100 animals using the circumferimetric technic. Thus, on the basis of assay precision alone, the circumferimetric technic is the better. An assay based on this technic, while certainly not of high inherent precision is feasible, being about as precise as the well-known assay for Vit. A in the depleted female rat.

Discriminant function analysis (Finney, 3) indicated that no additional information was contributed by use of the combination Y_1 and Y_2 over that obtained from use of Y_2 alone.

Summary. 1. The edema of the rat foot produced by serotonin injection as influenced

by aminopyrine has been quantitated circumferimetrically and observationally. 2. The data obtained by circumferimetry have been analyzed by means of response metameter,

$$Y_2 = 100 \log \frac{2 \text{ hr reading}}{\text{Zero hr reading}}$$

3. Results obtained by circumferimeter can serve as basis of quantitative anti-edema assay with inherent precision $\lambda = .57$. 4. Subjective scores either alone or in combination with circumferimetrically derived data are less satisfactory for use in this assay.

TABLE I. Y_1 and Y_2 and Varying Aminopyrine Dosage.

Doses of aminopyrine (mg/kg)	Response		N	
	Y_1	Y_2	Y_1^*	$Y_2^\dagger$
0	$2.23 \pm .17$	$5.37 \pm .38$	30	10
50	$1.90 \pm .17$	$4.10 \pm .38$	"	"
100	$1.37 \pm .17$	$3.47 \pm .38$	"	"
200	$1.33 \pm .17$	$2.98 \pm .38$	"	"

* No. of scores.

† No. of measurements.

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